BLOOD CLOTTING AND ALLIED PROBLEMS

Transactions of the Fourth Conference

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Edited by

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JOSIAH MACY, JR FOUNDATION CONFERENCE PROGRAM

FRANK FREMONT SMITH
Medical Director

As an introduction to the Fourth Conference on Blood Clotting and Allied Problems I would like to outline what it is that the Foundation hopes to accomplish by its Conference Program

We are interested first of all in furthering knowledge about blood clotting and to this end the participants in this meeting were brought together to exchange ideas experiences data and methods In addition to this particular goal however there is a further and perhaps more fundamental aim which is shared by all our conference groups This is the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and a very urgent one the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another channels must be established for the most relevant dissemination of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science is a serious obstacle to scientific progress Particularly is it so in medicine that the limited view through the lens of one discipline is no longer enough For example today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation At the other extreme medicine is certainly a social science and through mental health must be concerned with economic and social questions The answer then is not further fragmentation into increasingly isolated specialties disciplines and departments but the integration of science and scientific knowledge for the enrichment of all branches This integration we feel can be encouraged by providing opportunities for a multiprofessional approach to given topics

Although the fertility of the multidiscipline approach is recognized adequate provision is not made for it by our universities scientific societies and journals And perhaps the presence of other hindering factors must be admitted Partly semantic in nature they may also to some degree be psychological Admittedly it is oftentimes difficult to accept data derived from methods with which one is unfamiliar By making free and informal discussion the central core of our meetings we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers

Thus our meetings are in contrast to the usual scientific gatherings They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice For this reason we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting Our hope is that the participants will not come prepared to defend a single point of view but will take advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories

We have now thirteen groups functioning under the Conference Program The following topics are covered adrenal cortex aging biological antioxidants blood clotting blood pressure connective tissues consciousness cybernetics infancy and childhood liver injury metabolic interrelations nerve impulse and renal function When a new conference is organized the Chairman in consultation with the Foundation selects fifteen scientists to be the nucleus of the group and every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting but for the purpose of promoting full participation by all members and guests attendance at any meeting is limited to twenty five It is inevitable that in no topic can we possibly include more than a small fraction of the key investigators in the field and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include

The transactions of these meetings are recorded and published This is done because the Foundation in addition to its interest in the problem of communication between scientists believes that conveying to those in other fields who are concerned with science

the essential nature of scientific research is also an important problem in communication Logic is a vital aspect of science but equally essential is the intuitive or creative aspect Research is as creative as the painting of a portrait or the composing of a symphony Although logic is of course necessary in order to rearrange to test and to validate research thrives on creativity which has its source in unconscious nonrational processes Unfortunately however in the finished products which are presented to the world through research reports this integral part of scientific endeavor is shriveled by the cold white light of logic By preserving the informality of our conferences in the published transactions we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays

METHODS FOR DIRECT INVESTIGATION OF FACTORS LEADING TO THROMBOSIS

MELVIN H KNISELY

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THE EASIEST THING for me to do is to outline possible relations between *some types of sludge* and thrombosis This involves a summary of experiences gained over a twenty year period of almost daily microscopic study of many parts of 5 500 living animals

In healthy normal animals the red blood cells are not agglutinated They do not stick together In fact they repel each other slightly and in carefully handled tissues even rouleaux are completely absent White blood cells do not stick to healthy normal endothelial linings I am not so certain about platelets The papers by Dr E R Clark and Dr E L Clark(1) should be read to learn what they have seen at very high magnifications

The flow of unagglutinated blood is laminar or streamlined and this is important in terms of fluid mechanics because it means that for every increment in pressure we get the maximum possible increment in rate of flow of blood This translated into physiology means that every available amount of pressure is used effectively toward maintaining rapid forward flow of blood in capillaries that is toward the rapid transportation of oxygen and glucose to the cells of tissues

In healthy animals and people small vessels do not leak fluid rapidly enough to cause microscopically detectable hemoconcentration of the passing blood or tissue edema However some tissues such as the spleen and liver are exceptions(2 3 4)

With regard to a criterion for sufficiently rapid flow the rates of flow in vessels from 60 to 120 micra in diameter are so rapid that individual red cells cannot be seen

One has to know the *normal* in preparation for recognizing the abnormal and also in preparation for therapeutic testing

Up to the present time agglutinated blood or circulating sludged blood has been seen in approximately 5 000 human beings We

know some of the mechanisms whereby sludge damages the body One is the resistance of the sludge to its own passage through the terminal arterioles and capillaries which forcibly reduces the rates of blood flow through all open vessels of the body Also the specific factors in the flow characteristics or as the people who work in fluid mechanics would say the rheology of sludged blood are much more complicated than has even been guessed A few of these factors may be stated here

Masses of sludge stick together they rub on each other and rotate a great deal Energy is dissipated in giving angular velocities to masses which are circulating and rubbing against each other Thus the energy of the arterial pressure is wasted and the lost energy cannot be used to keep blood flowing hence the streams slow down In our laboratory we now have a man working on these problems which constitute a whole new subdivision in the rheology of abnormal blood

Under some conditions at least agglutinated red cells have coatings on them and these coatings cause the red cells to adhere to the stationary phagocytes of liver and spleen This has been directly observed in the edge of the liver of rhesus monkeys with *Plasmodium knowlesi* malaria

There is *settling* and *sedimenting* of masses of agglutinated blood cells out of the moving blood plasma to the lower side of vessels *during life* and that is what we will discuss today

Some small vessels are plugged with bigger masses of sludge These plugs act like small dams blocking some of the passageways from arteries to veins thereby slowing the flow in larger vessels When this flow is slow enough then masses of sludge begin to settle in the large vessels

As the blood volume goes down the vasomotor system almost certainly initiates intermittent prolonged controlled spasms of arterioles in a selected series of organs

Our experience to date indicates that these spasms begin first of all in structures made out of collagenous connective tissues and then progresses into striated muscles Thus the rates of flow of blood through the striated muscles and connective tissues may be reduced to zero for long periods of time during low blood volume

Figure 1 is a diagram of the inferior *vena cava* of a rhesus monkey with malaria The monkey had been spreadeagled" on a board

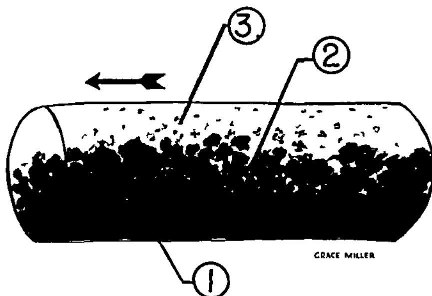


FIGURE 1 Diagram showing settling of masses of sludge in the horizontal *vena cava* of a monkey with malaria
1 Thickly packed masses stationary on the bottom
2 Masses moving slowly forward
3 Plasma with but few cells passing along the top of the vessel

for several hours and a celiotomy done to expose the inferior *vena cava* for observation The figure shows that the top third (labeled 3) contains fewer red cells and some white cells And as you can see the bottom of the vessel is thickly covered with settled sludge (labeled 1) This has a number of implications beyond thrombosis and was the starting point for our own study of this set of phenomena For instance the parasitized red cells sitting on the bottom are nurtured carefully and are completely prevented from being carried to the stationary phagocytes of spleen and liver Thus one defense mechanism of the body is prevented from destroying malaria parasites in this settled sludge

The following studies are parts of the thesis of Dr Louise Warner(5) which can be borrowed for study Figure 2 shows the mesentery of the frog held in the vertical plane The microscope is tipped over and one looks horizontally through it at the mesentery There are other ways of mounting and illuminating the tissues all described in her thesis Figures 3 to 7 show various stages of sludge formation



FIGURE 2 A general view of the experimental arrangement Note that the mesentery is vertical and that the microscope is horizontal

One of the main points that I want to make here this morning is that I do not believe that we can hope to study pathologic physiology on an eight hour day basis

One of the most difficult things that I know is to try to estimate in advance the rate at which to run an animal experiment in order to parallel the pathologic physiology which occurs in a man

If a man is put to bed he may be in bed for weeks days months whatever you want

The behavior of scientists is largely governed by social phenomena when people eat when they get up in the morning when parties come and so on so sooner or later experimenters have to throw away everything in life but the experiment do that and nothing else or do the work in teams

Figure 8 is a strobe flash picture of an individual capillary It is a capillary containing one of the largest red cells known to man The living red cell is 70 micra long Ordinary capillaries are wide



FIGURE 3 Figures 3 to 7 inclusive show masses of sludge settling to the lower sides of vessels which are horizontal or nearly horizontal We now believe that this is a necessary step in the formation of some kinds of venous thrombi One value of the method of study is that it permits steps in the processes of the formation of thrombi to be studied directly Now a great many new principles of these pathological processes can be studied in detail

enough so that red cells can go through in single file White cells can also be seen

I S Wright What species is this?

Knisely *Amphiuma tridactylum* It has the largest red cells known to man

Link Is there a common name?

Knisely It is a cousin to a frog or mud puppy The animal looks like a snake and may be from one to three feet long They are quiet animals seldom found by untrained people and seldom have a local name

These five figures together with figures 8 through 11 are a selection from a series of photographs by Mr Fritz Goro of *Life* magazine part of which was published in color by *Life* magazine in the issue of May 31 1948 It is a privilege to acknowledge the assistance of *Life* magazine and Mr Goro in making these photographs and for permission to publish them



FIGURE 4

Most of the knowledge about *amphium tridactylum* has been collected by Dr Baker in the Biology Department of one of the Memphis schools May I tell you a fantastic thing? In the *amphium tridactylum* the white cells are enormous When you are watching two or three of them one will come up and carefully *step over the other* They do not always drag pseudopods over each other They walk over each other stepping high something like an *octopus* It is a fantastic sight

When white cells begin to stick on the inside of vessel walls they make the lumen narrower The equation of Poiseuille which we ordinarily use for blood flow through narrow tubes indicates that the rate of flow *is proportional to the fourth power of the diameter* — other things being equal

So if we cut the effective internal diameter of a vessel in half we will get but one *sixteenth of the previous forward flow* And one sixteenth of the rate may be *enough* to permit masses to sediment from the flowing of blood



FIGURE 5

Some people have felt that sludge blood did not occur in arteries but just in veins However a photo flash taken in a cat s mesentery* clearly shows that masses are present in the artery too Thus this sludge resists its own passage through terminal arterioles

Another slide shows that the masses in the vein are smaller than the masses in the artery Blood pressure energy is being used to crush masses and force them down through cone shaped arterioles Thus the arterial pressure energy is being dissipated into factors which do not contribute to a forward flow

We have *increased the peripheral resistance* by changing the blood to a sludge That is a simple way to say it for physiologists

Figure 9 was made in a frog at the beginning of one experiment Tissues are mounted up and you see them horizontally These are frog capillaries The red cells are agglutinated a little – maybe two

* Slide not submitted for publ cat on –*Editor*



FIGURE 6

or three red cells sticking together in small masses but not more than two or three red cells to a mass

Note the upper and lower edge of the endothelium of this vessel

Figure 10 shows the vessel in spasm This vessel is packed full of red cell masses

Now, this vessel *is completely plugged Notice that this vessel* segment is *beginning to bulge just like an automobile inner tube* which is blown up a little too much

Figure 11 shows a vessel solidly impacted *now the red cells* in the impacted vessels are beginning to go through a series of colors which are indicative we think of the disintegration of the hemo globin of the trapped red cells

With so many red cells settled out the *circulating blood* has a very low red cell count This condition existed for several hours before the animal had a failure of heartbeat or failure of respiration and died"



Figure 7

By ordinary gross methods one would say the whole animal was alive but actually as a histologist looks at it there were great areas of the animal that were already dead long before the heart stopped beating

We have tried also to learn as much as possible about the specific factors which will determine whether settling occurs and whether the settled masses cement together to form a thrombus Further I am sure we have not more than begun this kind of study

In this respect there are approximately eight pages in the summary of Dr Warner's thesis listing what has been learned so far I will list a few high points

Settling has been seen in the *inferior vena cava* in monkeys so we know it can happen in fairly large vessels

In vertically suspended mesenteries — once these masses begin to settle the process goes on in all vessels of a) the same diameter

b) having the same linear flow rates and c) which make the same angle to the horizontal

There seems to be a critical velocity of forward flow above which masses of a given size remain in suspension And if the rate of flow becomes less than that critical velocity the masses begin to settle out

The slower the blood flows in any given vessel the sooner the masses begin to settle to the lower side of that vessel

The plugging of the tips of arteries and capillaries with the larger sized masses of a sludge stops the blood flow through the tips of those vessels and thus slows the blood flow in the stems enough to permit the beginning of settling in the stems of the vessels

Another important factor is spasms of vessels Sometimes in a preparation where we know settling should occur the circulation may go on for hours with no settling at all In such cases settling can be made to occur by producing a small hemorrhage Then many small vessels go into spasms and settling begins immediately

Furthermore the lower the red cell count in a given set of vessels or in the entire animal the more freely the agglutinized blood cell masses can and do settle to the lower sides of vessels This is in complete agreement of course with our knowledge of sedimentation rate in the test tube

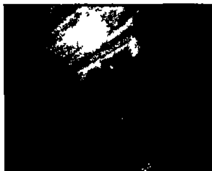
The larger and more dense the individual mass of a sludge the more they are apt to settle If one has a sludge with masses of mixed sizes the large ones settle out first When two or more masses of subcritical size stick together and form a mass above the critical size which is often observed the new large mass suddenly begins to settle These processes go on rapidly so that when there is a change in the mutual stickiness of masses small masses suddenly stick together making large ones and showers of masses begin to settle out quickly

The angle the vessels made and whether they are straight or curved are important factors in determining whether settling occurs and at what flow rates When the settling has begun it begins first and proceeds most rapidly in vessels where the flow is slightly uphill and starts later in vessels more horizontal and much later or not at all in vessels which run downhill I think this is most important for consideration by the clinicians here

FIGURE 8

FIGURE 10





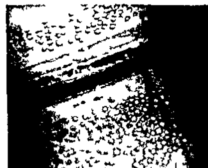




FIGURE 9

FIGURE 11

FIGURE 8 Strobe flash p cture of the capillary of an Amphiuma tr dacytium

FIGURE 9 Wh te blood cells st ck g to the cap llary of an Amphi ma

FIGURE 10 Photoflash picture of sludge in the artery of a mesentery (From a cat) Note that the ma ses in the artery which is the narrower vessel are larger than the masses in the vein This p cture pro es that the masses must be forcibly smashed to get through the artery

FIGURE 11 P ssage of sludged fragments nto arterioles (From a frog) The tip of the triangle is just over the branch of the artery The masses in it are mo ing toward the upper left In the top of this same scene are a number of small vessels filled w th a clear homogeneous redd sh transparent mater l These vessels are sol dly impacted with masses of sludge

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You remember years ago patients used to be put in bed with pillows under the knees, and obviously in this case the blood is going uphill in the veins.

Perhaps parenthetically I should point out that agglutinated blood, or sludged blood, now has been seen in something more than 5000 human patients, patients with different diseases, and so if the physics is the same in patients as in experimental animals we can expect settling to occur in human patients.

One more thing. The sludge may not contribute in any way beyond what I have said, to the formation of thrombosis. We might take the position that sludge is incidental but not contributory to thrombosis but certainly thrombosis occurs in circumstances where we know sludge is present.

I S Wright Would you repeat that?

Knisely As members of a critical audience you might say "sludge has nothing to do with thrombosis" and in our current state of ignorance you could not be proved wrong. But thrombosis occurs following surgical injuries and may occur in the pelvic veins following childbirth. And in each of these conditions we do know that sludge is present. Hence we can be sure at least that sludge does not prevent thrombosis.

Barker Always?

Knisely In all experimental animals that I have tested,(6) and in patients who have been studied by those in Chicago(7 8) and in the study by Zilliacus in Finland(9) who has been studying the development of the sludge in women during delivery, in all such cases sludge has been present.

Alexander Does it vary on different days after trauma or does it come at a more specific time, e g immediately after trauma?

Knisely I can give partial answers but certainly not complete. The sludge is developed immediately following trauma.

We have taken motion pictures while crushing a tissue and sludge begins to be formed in some cases in as short a time as one-quarter of a second after the crushing instrument is removed. The time was measured by counting the motion picture frames between crush and the start of sludge formation.

I have seen human beings who have been crushed badly in one place build up a sludge in the whole circulatory blood.

That "rheology" we will have to work out ourselves

I S Wright Can you tell us precisely how you decide that a group of cells is passing along as a sludge rather than as a group of cells that just happen by chance to be passing along together at the same time?

Knisely If you have a very high magnification you can see individual red cells However usually we do not have to use high magnification If you are looking at a mass and see that the whole mass turns over tumbling as a unit you know that real forces are holding the red cells together You can watch the turning and tumbling of individual masses quite easily Is this the answer?

Tocantins Well I have difficulty in deciding just what constitutes a sludge and what the criteria are for recognizing a sludge is it the size of the mass its outline its stickiness?

Knisely If you had the blood altered so that all red cells were sticking together in pairs you would then have altered the whole rheology of the system Also if the pairs were held together with a coating precipitate they might be eligible for phagocytosis in the liver and spleen

For example if there is a fork in a vessel and one red cell comes down it will fold over very easily If red cells are together in pairs the couplet is less flexible The two cells reinforce each other and offer more resistance to being folded particularly when entering narrow vessels

Let us say a large red cell will fold up like a pancake and be pushed through a narrow capillary Two or three will not do that as easily in fact the vessel may have to bulge to permit passage This consumes energy which comes only from blood pressure

Tocantins The stickiness of the mass would also be a restriction?

Knisely Some masses are sticky and some not In the sludge following trauma in dogs the masses are naturally quite sticky In sludges following severe burns in dogs the masses are not sticky and do not stick to each other

Edsall I should like to be sure that I understand just what you mean by laminar flow In the case of a Newtonian fluid laminar flow means that the layer of liquid immediately adjoining the vessel wall remains at rest Away from the wall the flow velocity gets progressively greater as the center of the vessel is approached?

H P Wright It persists for how long?

Knisely Sometimes for days

Tocantins Would you please detail the criteria whereby a sludge may be identified?

Knisely This involves the definition of the word sludge which is important and is apparently bothering the biologists a little The term sludge is a word used by engineers You will find it in the large Webster s Dictionary It is a term used to describe the physical characteristics of a fluid containing suspended particles The fluid medium may or may not be a truly Newtonian or viscous fluid and the particles may be solids semisolids and elastic bodies or plastic bodies The physics of the flow of a suspension of this kind is quite different from the physics of the flow of a simple viscous fluid I would say that if the blood was changed so that all the red cells were stuck together in pairs we would have the simplest and least damaging type of sludge

Tocantins This is similar then to what happens to plain blood standing in a sedimentation rate tube Would you call the pseudo agglutination of the red blood cells a sludge?

Knisely You put me in a difficult situation because I differ from most people in my opinion of what takes place in a sedimentation rate tube

In terms of physics that would be a sludge yes You have a solid suspended in a Newtonian or viscous fluid or approximately that type of fluid

There is extensive engineering literature dealing with the pump ing of sludges Until about 1939 the physical studies of sludge pumping were carried out in pipes never less than three inches in diameter and more commonly about two feet in diameter

There is a whole division in the science of physics dealing with problems of pumping such mixtures An introduction to part of that literature can be obtained from the Annotated Bibliography on Sludged Blood which is now in press It will appear in *Post graduate Medicine* during 1951 The physics or rheology of sludge has been partially worked out but no one has dealt with the prob lems which arise when sludge is pumped through vessels which themselves are bulged by the individual masses as they go through the vessels

Edsall I think that seems to be a good general description of what is going on

Knisely So far I have said that we have masses of the same size If we put into the system a large mass it will go rolling and tumbling along completely tearing up all of the laminar systems

Another thing which we are worrying about now is that some of these masses behave as though the center of the mass is not at the geometric center so they rotate in most irregular fashions and because of that cause a pounding motion The summation of these irregular rotations should be a tremendous energy dissipating system

Alexander May I ask whether one of your prerequisites for a definition of sludging is agglutination of the red cell?

Knisely I think so Let me say things historically as they happened in the laboratory At first we used the term intravascular agglutinization of the circulating blood and when we continued to discuss it saying every time "intravascular agglutination of the circulating blood somebody finally said let s cut this term down to simple talk" and the answer was sludge! And so the word "sludge was used in the laboratory a long time before any thought of publication

We had a colleague who was a Latin scholar and he coined the term atoporhemia He had put it together from Latin and Greek roots and proposed it for serious consideration

I would like to see anybody make laboratory workers use that word He proposed it but nobody ever used the term The popular word was sludge and that is all there was to it Fortunately of course the whole engineering literature is written around the word "sludge" and so I think we ought to know as much about the properties of the sludges as the engineers know Perhaps we can just move that knowledge over into the biological world

Ferguson The presence of the corpuscles in blood alone suffices to alter the rheology in a way that to me suggests that the applicability of the idea of sludging in its simplest form is merely the tendency for a sedimentable portion (e g corpuscles) to modify flow relationships in a liquid I gather however that Dr Knisely wishes to restrict the term to additional problems which arise when the formation of cell *masses* occurs and causes other types of blood flow patterns than those occurring under normal physiological conditions

In this case you cannot apply that definition strictly and I wonder in just what sense you would interpret the word laminar in describing the flow of normal blood

Knisely In a strictly Newtonian fluid with the individual molecules of the fluid free to move on each other the accepted ideas are that those on the inner surface of the tube remain stationary while those of the next layer inside move at a low speed with the next layer going a little faster Furthermore the plotting of these rates gives a curve which approximates a parabola Certainly some sets of molecules of blood plasma are stationary on the internal surface of the vessels However plasma consists of molecules of many different sizes and shapes so the plasma part of the blood is not going to behave according to our previous definition of a pure Newtonian or simple viscous fluid

Let us introduce a very simple system in which we have free individual unagglutinated red cells We put in just enough red cells so that we have three linear rows when seen through the microscope We really have a central core going down the axis of the vessel and one concentric lamina of red cells around it Now if we had a layer of molecules here they would be carried along on the outside of the red cells

In the case of unagglutinated blood cells we have the maximum number of individual — I am not going to use lamina here but the word shells Let us define a shell as a cylindrical configuration of red cells or of red cell masses in flowing blood As observed in sludged blood red cell masses are carried along in concentric cylindrical configurations and these cylinders exhibit a special type of laminar flow However in agglutinated blood there are fewer concentric shells in the laminar system With agglutinated blood the red cells may be stuck together in very large masses and actually there may be a row of them bumping along in the middle of the vessel Also in agglutinated blood the number of shells depends upon the sizes of the individual agglutinized masses

Further there is useless dissipation of energy in this system Each mass in the sludge is subjected to forces which tend to rotate it the side of the mass nearest the vessel wall going forward more slowly than the side toward the center of the stream This imparts an angular velocity to the mass which now turns and tumbles as a result of these forces Thus the force of arterial pressure is used up the last part then is no longer available to maintain the forward flow of the blood In brief this wastes blood pressure

Knisely This leads to a third point In some cases at least there is certainly a sticky precipitate on the outside of the cells In the case of the precipitate on the red cells of rhesus monkeys with malaria you can pull it off with microneedles like chewing gum

H P Wright Are you differentiating between agglutination and adhesiveness? This should be clarified Agglutination is as it were a reversible reaction but I do not believe that a true adhesion is biologically reversible

In other words do your sludge masses remain as the same mass or are the cells changing? Are they moving from one mass and on to another? In true adhesion

Knisely Now you have opened up two arguments

We thought about two kinds of masses in sludge one is the basic mass which does not break up as it goes through arterioles and capillaries

H P Wright You mean they are sticky?

Knisely Some are and some aren t Let me offer the arguments The only specific factors in the definition are that as these masses do not break up as they go through arterioles and capillaries they have enough internal force to hold them together against whatever force is brought to bear upon them by the circulation

H P Wright Which may be just capillary attraction between the different surfaces

Knisely That isn t our concept because if the surfaces of the red cells are in contact with each other the negative charges are enough to push them out of contact In fact in perfectly healthy animals they do not stay in contact — the red cells repel each other

For instance if red cells are coming down a vessel in which they have been forced into contact by going through a leaky vessel segment then the red cells will separate again as soon as they enter the larger stream

Now in a sludge the red cells in a mass do not separate Also in some sludges the masses have precipitated material holding the red cells together In other sludges our current methods do not permit us to identify materials between and around the red cells in the masses but these masses withstand rather strong compressing forces without breaking up Masses may go through arteriole tips

Knisely I agree with you

Edsall Is there any evidence of a factor in the sludge blood which tends to agglutinate the red cells? For instance if a little of the plasma from sludged blood is added to another sample of blood will the red cells clump faster? It is known especially from the work of Fahraeus that an increase of blood fibrinogen will do just that Dr John Gibson and Dr E S Buckley Jr of the Harvard Medical School have applied this principle by adding extra fibrino gen in order to produce rapid sedimentation of the blood freshly taken from a donor so that cells could be separated from a plasma without using centrifugal force Other substances will work also if they are composed of elongated molecules — dextran gelatin and certain synthetic polymers The only property these seem to have in common is that they are all elongated molecules However as far as I know fibrinogen is the one which is known to be physio logically present

Knisely At this point my knowledge comes only from observa tion of patients and from more or less physiological experiments in the laboratory without *chemical* identification of the materials on the surface But I would like to put in the record that there are at least three ideas One is that something has been peeled off the red cells as a layer is peeled off the outside of an onion In such a case we would be dealing with an inner newly exposed surface and that would change the red cells

Another idea is that there is a change in the electric charge on the red cells In my state of ignorance I would assume that if you had *many* changes in the charges certain proteins would begin to stick to the red cells The third

Edsall One would certainly think of electric charge as a major factor in these things The awkward thing to explain is that the red cell I believe is negatively charged and so is the fibrinogen mole cule at physiological pH So the natural desire to think in terms of a simple electrostatic mechanism does not seem to fit the facts as far as I can see

I should guess that there must be some attractive forces that cause the fibrinogen to stick to the red cell but apparently the electrostatic factor should be a barrier rather than a help

I believe it is correct that if cataphoresis is applied to red cells under physiological conditions they move as if they were negatively charged

Edsall It would not be a chemical experiment You would take the plasma and test its action If the result were positive the chemists could then try to isolate the factor that produced the effect

Knisely I would say that it should be done and try to excuse ourselves by saying that we have been busy on other aspects of the problem

Zucker Do you find the red cells in clumps if you draw blood from an animal which shows sludging and observe it under a microscope?

Knisely It is possible In some cases of monkeys with malaria Dr Bloch was able to withdraw blood with the masses remaining as clumps Most of them disintegrated when they were put in Ringer s solution We have done this under dark field and photographed the masses

This is a key point Some people have felt that because they do not get masses *in vitro* the phenomenon of sludge *in vivo* wasn t real

In terms of pathologic physiology the significant thing is that these masses have sufficient internal strength to withstand the forces brought to bear on them within the vascular system and not be broken up But we are still left with the problems of how to get them out so that we can study them *in vitro*

There is some accessory information here as follows If one goes into a hospital and watches people counting red cells one often times finds them having a desperate time because the red cells they are trying to count are all in little masses on the slide

I wander into the hospital late in the evening as though I were a patient who got in the wrong room and say What are you doing?

Oh the technicians say We are counting red cells"

What are you doing that for?"

"What a silly question The doctor said we should count them"

What are you shaking this thing for? I ask

Well you have to shake the pipette to keep the red cells from sticking together

and actually bulge the arterioles This probably means that some thing more than surface tension is holding the red cells together

The other kind of mass is the great cluster of these basic masses The basic masses never become very large The biggest ones that I know about are probably between 25 and 50 micra in diameter at the time they are going to terminal arterioles certainly not more

Alexander After sludging supervenes have you any data on the length of time a given column of blood remains completely stagnant in time intervals?

Knisely Anything can happen A column of blood may stop or the whole thing may go at one tenth the normal rate The mass may plug the tip of an artery

Alexander And that would remain stagnant for hours or minutes

Knisely This happens all over the body in traumatic crushing shock in dogs and in burn shock in dogs Some plugs may remain stationary for but a short time others may remain until and after the death of the animal It depends upon the sizes of the masses the degree of internal rigidity of the masses and how sticky they are to each other

I S Wright Dr Edsall raised a question which you might be willing to answer in greater detail Have experiments been carried out in which serum or plasma has been injected into another animal and if so will this produce the phenomenon of sludging in the recipient animal?

Edsall I really had in mind an *in vitro* experiment of a similar sort that is to take a little blood or some washed red cells from a normal individual add to that in a test tube some of the plasma from an animal in which the sludging is occurring and see whether the plasma from the sludging blood would have any accelerating effect on the sedimentation rate of the normal red cells

Knisely *We have done neither the in vitro nor the in vivo* experiments

I S Wright It would be an interesting thing to do

Knisely But I think it should be done by people who know proteins It shouldn t be done by us because we cannot make the interpretations properly

I S Wright Well you can determine whether sludging sud denly takes place

while and cement together without cementing to the vessel wall At still other times they will cement together and cement to the vessel wall sometimes not cementing very tightly as a result of which the whole mass will break loose and go away Again they cement so tightly that no ordinary procedure will break them loose during the experiment until after the death of the animal

With regard to the methods of study Dr Warners experiments are especially important because the methods permit us to get living tissues and living vessels out in the open and study them while the processes of thromboses are going on

I think it would be important to test the effects of the anti coagulants on whether the mass cement together or not or do they cement to the vessel wall And it would be important to test the effects of drugs to break up the sludge and find out if a thrombosis occurs in the absence of a sludge We dont know that

Nothing has been said so far about the "propagating thrombosis" A sample type of thrombosis has been studied the thrombosis which is formed by having masses settle out and all stick together after ward One of the problems is to find out as many different kinds of thrombi as we can and begin studying *each one by itself* aiming ultimately at complete prophylaxis for all the kinds we find

One more thing about which I am really disturbed When we as *experimenters* go to study thrombosis we take an animal which is in reasonably good health and do something to him and say get a thrombus Under what conditions? That experiment may parallel what happens to a man who has a serious automobile acci dent lets say a traumatic experience or surgery for a hernia when there is nothing wrong otherwise But there are a great many thromboses developing in persons who have chronic diseases and I am worried for fear that study of what happens in a relatively well animal over a short period of time may not tell us too much about what goes on in people who have much much more complex pathologic physiology before we find a thrombus

And so I would like to propose to you a most radical idea Let us set up equipment to study what is going on in human beings while celiotomies are being done It is now socially acceptable to do an exploratory celiotomy when the physician is hunting for something about which he already knows The only variation I am proposing is that he hunt for and study some things about which he does not yet know

How long do you shake it so that the red cells will not stick together again when you stop and then if you shake it that long why don t you shake it twice as long as that? Usually there is no answer to this question

Edsall You referred to clumps breaking up in Ringer s solution Now suppose you break up a clump in Ringer s solution and then centrifuge the cells down and resuspend them in plasma from the sludged blood would they clump again?

Knisely As I said previously there are three guesses as to the changes concerning the red cells in sludge

One is that the outer side may be peeled off another is that a charge may have developed on them and third some sticky pre cipitate may be on them

The point I want to make is this it seems to me that it will be necessary to examine a whole series of diseases and to examine the cells to find out whether they have only blood clotting proteins in them or whether they also have the specific immune proteins stuck on them

One reason I have been so careful not to come to a conclusion is that if we decide it is a question only of blood clotting we would discard the ideas about the proteins of immunity For this reason each kind of sludge should be examined both by men who know the blood clotting situation well and by immune chemists but certainly not by us because we are only physiologists

Dr E H Bloch has begun some of this work and has found that there is a different rate of electrical mobility between red cells from healthy blood and from some kinds of sludged blood If a man had measles then typhoid fever and later tuberculosis the coating material might be different than if he had first tuberculosis then typhoid and then measles

There are specific characteristics of sludges Masses are different from case to case they are different from one patient to another

I S Wright Dr Knisely will you conclude your summary so that this interesting subject can be opened for full discussion?

Knisely To go back to the sludge and settling and thrombosis sometimes masses will settle to the lower sides of the vessel and not cement together They are quite loose from each other and after any interval the forward flow will increase and they will all be carried away At other times masses will stick together for a

Lord Kelvin said If you understand something you can make a model of it And some time later I think it was J B S Haldane who said that If you make a model of something you understand the model

Part of our problem is to get into living men and find out what is really going on in a host of different circumstances At least we could put a flashlight on the inferior *vena cava* and see whether we get settled masses after prolonged surgery particularly if the patient lost some blood so that he might have spasms of vessels in the legs

Dr Best and his colleagues prepared a motion picture for the purpose of studying a series of conditions and I think Dr Best ought to describe it*

Best The film is an old one which many of you have seen and now may have only historical significance At first we attempted to make a platelet plasma by differential centrifugation and then watch the platelets settling out on the glass surface The pictures were not very good because the diffraction rings could not be eliminated Then Dr James Craigie helped us with the photography and as the film shows the formation of pseudopodia is clearly seen In some cases one can observe what might be called the birth of a thrombus in that two platelets stick together The pictures seen here were taken using an oil immersion objective In the colored part of the film a transparent cell was interposed between an artery and a vein This extracorporeal shunt is really an arteriovenous shunt By watching the interposed cell one can determine how soon a thrombus forms

It is found very definitely that the prevention of platelet agglutination lagged considerably behind the change in the clotting time of the blood that is the blood became uncoagulable and yet there was some time before the platelet agglutination was decreased by the heparin

For teaching purposes we speeded the film up so that it looked more dramatic This should of course be shown simultaneously with another film in which nothing can be seen — this would be the effect of heparin

In the monkey and dog and with more difficulty in the rabbit

* At this point Dr Best showed a film on the formation of platelet thrombi Part of his commentary follows —*Editor*

One trouble with the *in vivo* methods (and it is not all a matter of the degree of microscopic magnification) is the inability to distinguish fibrin formation Gross clotting according to the evidence of pathological histology would seem to come somewhat later than cell coherence and adherence and of course the hemostatic and obstructive effects of a thrombus owe a lot to the fibrin meshwork supporting and buttressing the cell mass What we would all like to know and do not know as yet is whether invisible films of fibrin can deposit on injured endothelial surfaces and on the surfaces of circulating erythrocytes to play an essential role in both the sludging and thrombus formation phenomena We have long been aware that a slight rise in fibrinogen level in the plasma facilitates the erythrocyte aggregation which so largely determines the RBC sedimentation rate *in vitro* Anticoagulants being used in this test it would seem that fibrinogen rather than fibrin is the active agent but I m not sure whether we can be certain of this since the conditions at the surface of an erythrocyte could conceivably permit of a trace of fibrin formation even though such is not detectible in the plasma One is on highly speculative grounds here however I would like to ask Dr Knisely whether he has injected fibrinogen into the circulation to see if this alone can evoke sludging? Does he also have any data on plasma fibrinogen levels before and after extensive sludging? Physiological response of the liver to raise the fibrinogen level as one aspect of the general reaction of the body to the noxious influence would need consideration here and might very well mask or even reverse any minor local loss of fibrinogen supposing this is a factor in erythrocyte sludging

Knisely Dr Seymour Gray injected electrophoretically purified beef fibrinogen into guinea pigs and it caused a profound sludge also changes in the surfaces of the blood platelets This was recorded in motion pictures but not published

We have no data on fibrinogen levels before and after initiation of a sludge They could be obtained though

I S Wright Dr Barker would you like to continue this discussion?

Barker I think we are indebted to Dr Knisely and his observations on sludged blood for giving us a new concept of circulatory pathology From what he says sludging occurs frequently following operations childbirth and tissue injury and during infectious diseases and the agonal period All of these conditions also may be

flow control in the capillary and small vessel systems which is well validated *and of great practical application to such problems as* have interested Dr. Knisely and his colleagues. I do think that there are many points of integration between the New York and South Carolina schools which it would be highly profitable to explore but I shall not attempt this now. Looking back on our observations I recall many instances of red cell masses, especially in stagnant capillaries, which now might be regarded as the phenomenon which Dr. Knisely terms "blood sludging."

Our interests at these Macy Foundation Conferences rather raise the question whether there are common factors between the blood sludging phenomena and the clotting and thrombosis problems. Just as it is clear from Dr. Knisely's data that factors such as lessened blood volume, diminished viscosity, vasoconstriction and slower rates of flow, etc., which contribute toward sludging must nevertheless have a more specific factor or factors to cause the sludging itself, so it would seem evident that sludging predisposes a true thrombosis with the associated stagnation and anoxia and other factors. Hence the key question is what these more immediate *factors may be. Endothelial injury certainly seems to be one of* them. In our micromanipulation experiments by more vigorous pricking with the needle we were able to alter the inner surface of a single endothelial cell and cause the appearance of that quality of stickiness which trapped passing *blood cells* and *built* up a cell thrombus.* We did conclude that the type of cell participating had a lot to do with blood flow and with the individual coherent qualities of the surfaces of individual cells and cell types. Thus with *slower* rates of blood flow the more numerous erythrocytes participate almost exclusively. When I snapped the photomicrograph a single leukocyte had temporarily attached itself to the distal pole of the red cell thrombus but it broke loose and was swept away a few minutes later. I would agree that Dr. Knisely's sludging and sludge settling phenomena would play a considerable part in the composition and lamination of thrombi.

Under *more rapid* conditions of blood flow in *mammals* as best shown by the movies made by Dr. C. H. Best and colleagues at Toronto with the glass capsule technique, pure platelet thrombi may occur. Under most thrombosis conditions one would conclude that all these factors come in and create a *mixed cell thrombus*.

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* See Figure 4 in reference 10 listed on p. 16

Clinically recognizable thrombosis occurs in less than 5 per cent of patients who have major abdominal operations I am sure that thrombosis acceptable as such to a pathologist occurs more frequently but I doubt that it occurs more than three times as frequently unless we broaden the definition of thrombosis to include any temporary stoppage of blood flow by clumping of erythrocytes This raises a question as to the importance of sludge which occurs in all postoperative patients in the pathogenesis of clinical thrombosis which occurs in less than 5 per cent of postoperative patients The same question has been raised regarding the importance of slowing of venous blood flow in the lower extremities during the postoperative period as a factor in the pathogenesis of thrombosis Several good investigations have shown that this slowing occurs almost always after major abdominal operations There must be another factor in addition to sludge and slowed venous blood flow which leads to the rather infrequent development of large propagative and potentially fatal thrombi in the ileofemoral veins I believe it was brought out at one of our previous conferences that anticoagulants which have been pretty successful in the prevention of thrombosis do not prevent sludging Is that still correct?

Knisely I would say that we have only begun to scratch the surface on that If we generalize on the few experiments thus far done we may get into trouble

The sludge formed in each species of host following each type of stimulus will have to be studied separately For instance a burned dog sludge or malaria monkey sludge or crushed dog sludge or a human crush sludge or a human malaria sludge — I dont think we are ready to generalize as yet

I S Wright I suggest that reference be made to the work of Laufman Martin and Tanturi at this point(11)

Barker To get back to the definition of thrombosis it appears that it may have to be broadened and that just as several factors may be involved in the pathogenesis of each type of thrombosis many factors may have to be considered in the pathogenesis of all types Coronary thrombosis as an example may be produced in several different ways I want to reiterate that regardless of its possible implication in certain well known types of clinical thrombosis Dr Knisely s work has certainly opened up a new and fertile field for investigation which may modify all our ideas about circulation of the blood in both normal and pathologic states We are all

complicated by thrombosis although much less frequently Perhaps the frequency may depend on our definition of thrombosis Un doubtedly the pathologist can find thrombosis in many patients where the clinician failed to recognize it The clinician is interested in all types of thrombosis but particularly in those that occur in coronary cerebral and peripheral arteries where there is frequently an extensive endothelial defect due to degenerative or inflammatory disease The clinician is also particularly interested in the thrombosis which develops in the iliofemoral vein and may lead to fatal embo lism or disabling chronic venous insufficiency where there is no known endothelial defect as a primary locus Is sludge a factor or a preliminary stage in the development of these two types of throm bosis? Many of these thrombi ultimately undergo partial lysis as well as partial organization However it seems to me that what is usually called thrombosis is a very different process from sludging where no fibrin or little fibrin is formed

I would like to ask Dr Knisely if the rouleau formation of the erythrocytes seen in the blood smear of patients with multiple myeloma or *polycythemia vera* indicates sludging or is a form of sludge

Knisely Insofar as I can give an answer let me take the polycy themia first I have seen a few patients with that diagnosis and their red cells were not agglutinated Their red cells were very close together and they turned over slowly as though the viscosity of the plasma was increased That I have seen *in vivo*

Barker Have you found sludge in any cases of *polycythemia vera*?

Knisely I have only seen two or three cases

Barker Have you found it in any cases of multiple myeloma?

Knisely I have never seen it If you find rouleaux on slides it certainly indicates we ought to look into the living person

Barker Many years ago clumping of erythrocytes with interven ing plasma spaces was observed by microscopic examination of nailbed capillaries in patients with both primary and secondary Raynaud s syndrome during the cyanotic phase of arteriospastic episodes This is a transient phenomenon which repeats itself many times in these patients but only rarely does true thrombosis develop in the capillaries or small arteries with resulting infarction of small areas of the skin

cold day even for a few minutes During these episodes their blood just stops circulating in the exposed parts but they have not developed clinical evidence of thrombosis during periods of observation lasting several months

I S Wright Some of our patients have too but some have progressed to gangrene and in one case death

Barker But cryoglobulinemia per se does not certainly lead to thrombosis does it?

I S Wright No but there may be other factors Furthermore only major thrombi can be recognized clinically but there are a much larger number present — which can only be demonstrated at autopsy Most of these never have clinical significance — 50 to 60 per cent of patients who are in bed for two or more weeks showed thrombi in their calf muscles if these were sectioned

Barker From the practical standpoint of mortality and persistent morbidity there is a considerable difference between the so called hidden or undetectable thrombi and the thrombi that are detectable clinically Do you agree? We have no way of knowing how frequently subclinical thrombosis becomes clinical thrombosis

I S Wright That s right We are going to ask Dr Jaques to discuss this problem

Jaques With regard to the problem that Dr Barker raised what do we mean by the word thrombosis and are we to extend the term to include sludging? If we do so extend it we are continuing a process that s gone on continuously ever since the word thrombus was first introduced in medical literature by Galen(13) "This humour immediately congeals not only outside the body but actually contained in its own surroundings and this congealing of itself we see terminate in the thrombus for by this term the Greeks name the congealed blood The term has been extended to include masses of platelets and in fact for a considerable period of time I think pathologists focused attention particularly on platelet masses as being of primary importance with the red thrombus being secondary so there is no reason why the term could not be extended to include sludging if it is thought advisable

In Dr Best s film we have seen one method for studying thrombus formation the extra corporeal loop technique Other methods that are available are the production of intravascular clotting by the injection of thromboplastin and thrombin the method which was

eager for more information on its significance in clinical medicine and what factors may make it reversible or irreversible

Knisely The belief of the people who have worked in our laboratory is that we are now only beginning to know enough about these things to *study* them and as far as I am concerned I am certain that we cannot provide information yet which will permit clinical interpretations

In each paper that we have written I have tried desperately to insist that this is the *beginning*

Now working with me is Dr Jorgen Weis Fogh of Denmark who started studying sludge under Professor August Krogh before the latter died Dr Weis Fogh is now making a correlation of his eight hundred sets of observations on patients and he tells me that in certain percentages he can correlate the presence of sludge with a low red cell count

Further in the monkeys with malaria we have seen the red cells coated and if the right kind of coating is on the red cell and if the phagocytes of the liver are ready to take up something the coated red cells are phagocytized causing anemia

Heimbecker and Bigelow of Toronto have been finding that following burns there is phagocytosis of red cells(12) In many diseases there are prolonged constant anemias and in some if you give a transfusion the transfused red cells disappear too — sometimes without any increase in free hemoglobin in plasma which makes us suspect that phagocytosis of agglutinated cells is a factor constantly draining blood out of the patient

I S Wright One of the more interesting diseases which has come to our attention at the New York Hospital recently is cryoglobulinemia in which sludge formation apparently occurs when the patient is exposed to cold — and this disappears when the patient is warmed up again

Barker Is there a tendency to thrombosis in patients with cryoglobulinemia?

I S Wright Yes there definitely is in some We have observed one patient who eventually died from multiple thrombosis In others *gangrene* of the digits and other areas have occurred

Barker We have seen some patients with cryoglobulinemia who developed severe symptoms every time they went outdoors on a

cold day even for a few minutes During these episodes their blood just stops circulating in the exposed parts but they have not developed clinical evidence of thrombosis during periods of observation lasting several months

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first introduced by Wooldridge(14) in 1886 and which has been investigated by many workers since then It is true that much of this work *has resulted from investigators in other fields finding* their particular tissue extracts toxic and who then went on to find that the toxicity was due to thromboplastin and thus they became interested in studying the intravascular clotting produced Mason (15) in 1924 and again recently has used this method in studies of intravascular clotting and the factors involved in the formation of the clot Another method which hasn t been touched on as yet is the method introduced particularly by Welch(16) of damaging the vessels by either mechanical means or chemical means We used this method extensively in our first investigations with heparin The first series of experiments consisted of exposing the jugular or radial veins of dogs surgically crushing the veins and inspecting them Such gross inspection of course simply told whether the vein was occluded or not However by fixing and using a standard histological technique the nature of the occluding mass could be studied under the microscope and from that we could get a picture of whether just typical blood clots had formed in which case a *uniform mass of red cells was observed in the vessels or whether* a white thrombus had formed as judged by the typical appearance of platelet masses with the typical layering observed in this type of thrombus In preparing for this discussion I went over some of the data of experiments we did back in 1934 and 1935 and I have several figures that I think may be interesting

The results are at this date perhaps more of historical interest than of any value per se but I think they bring out points pertinent to the present discussion I may say that some of this data was not published in our original papers

The first point is one of technique The textbooks give you the impression that damage to the blood vessels results almost 100 per cent in the formation of a thrombus The surprising thing in the experimental animal is how difficult it is to damage the vessel I think in this case it really means damage of the intima as this alone produces thrombosis For example in our first series shown in Table I we crushed a series of veins in dogs using a hemostat and in only one of nine was the vessel occluded and in one was there a slight deposit We then tried the technique used in our further work namely a cotton thread was passed through the lumen of the vessel and then the vessel crushed with a hemostat The cotton thread provided an agent on which the intima could

be traumatized Ten veins treated in this way were all occluded It didn t make any difference whether after crushing the thread was removed or not — the results were the same As shown by a number of early workers if the thread was left in place without crushing occlusion gradually occurred

Table I

Results of Crushing Veins With Hemostat

Technique	Time from procedure to injection	Number				Type of occlusion
		Total	Patent	Slight	Occluded	
Crush No Thread	1½ 23 hrs	9	7	1	1	mixed thrombus
Thread No Crush	1 hr 7 days	8	2	0	7	mixed thrombus
Thread Crush left	½-47 hrs	10	0	0	10	clot
Thread Crush Removed	½ 24 hrs	9	0	0	9	clot

Now I said that by inspection of sections of the vessel one could get some picture of whether a clot had resulted or whether deposition on the damaged intima resulted in the building up of a white thrombus With the technique of crushing on the thread in all cases observed there was a clot in the vessel — that is there was no evidence in section of platelet deposition However with the thread alone there was clearly observed a typical white thrombus in a part of the vessel and over that filling the rest of the vessel was a red thrombus

Table II I think brings out an interesting point because this was really at the basis of why we originally said that heparin prevented thrombosis (in the sense of a thrombus consisting of a mass of platelets) and that we had more in our data than just the prevention of clotting If the vessels were left in place for one to three and a half hours and heparin was administered so that it was effective for a portion of this time but not the whole period of time out of fifteen vessels six were clear three showed a slight deposit and six showed a clot In other words the results were just the

same as far as the nature of the occluding mass was concerned as in the controls Of course in contrast to the controls with no heparin administration there was a fair number that showed no occluding mass at all

On the other hand if the vessel were removed for examination four to forty eight hours after traumatizing the vessel and the heparin was effective for a somewhat longer period than one to three and one half hours then we found that out of seventeen vessels operated on while there are still a considerable number filled with clots quite a high percentage were filled with platelet thrombi

TABLE II

Effect of Heparinization on Nature of Thrombus

Saphenous or radial vein crushed on cotton thread with hemostats and then thread removed Single dose of heparin given intravenously

Examined in hours	Heparin Effective for	Number of Veins				
		Total	Patent	Slight Deposit	Clot	Platelet Thrombus
1 3½ hrs	½ 2 hrs	15	6	3	6	0
1 3½ hrs	1½ 4 hrs	6	5	0	0	1
4 48 hrs	1 6 hrs	17	1	0	8	8
7 days	70 hours*	33	26	0	7†	

* Continuous injection

† The figure 7 is inserted between the columns since differentiation was not made between clot and thrombus

Now if heparin were given either for the whole period before removal of the vein for examination or if the heparin were given for a sufficiently long period then a very large percentage of the veins were found to be completely free of any mass This was the basis of our original conclusion that heparin prevented both intra vascular clotting and thrombosis

This type of approach has also recently been used by Rabinovitch and Pines(17) in 1943 in which they traumatized the jugular veins of dogs I think this particular work should be drawn to your atten tion because Dr Barker mentioned that thrombosis is essentially an irreversible process Rabinovitch and Pines found that if the injection of heparin were delayed until the second day after the

trauma four out of five experimental animals showed patent vessels while the controls were all occluded

Barker May I make a correction I believe that some types and stages of thrombosis are irreversible but I did not state or mean to imply that all thrombosis was irreversible

Jaques I might add Dr Barker that it is partly our own thinking which is perhaps at fault I have heard Dr Best make the point that in the early days in order to have the idea of heparin therapy accepted it was necessary to suggest very strongly that heparin could not reverse the process Perhaps my correction here is more a correction of our earlier statements and thinking than a criticism

Barker Do you think that heparin itself has a fibrolytic or reversible effect on a thrombus that has already formed? This is a question which has been asked many times

Jaques My own opinion is that heparin has no direct effect on fibrinolysis If anything it probably inhibits this but I think heparin and anticoagulants in general by maintaining blood flow through a thrombosed vessel by preventing further extension of the thrombus allow the fibrinolytic mechanism to act in that area

Barker Does it act faster than it would have if anticoagulants had not been given?

Jaques Yes much faster than they would otherwise I think we now are coming to the view (although it still requires a great deal of experimental evidence) that the fibrinolytic mechanisms are something that are effective *in vivo* and probably are effective at a level or at a rate which is much greater than anything we have conceived of in the past Hence heparin by maintaining some blood flow through preventing the extension of the thrombotic process will allow a rapid reversal of the thrombosis *

Ferguson I should like to comment on this question of thrombolysis after heparin and heparin like therapeutic agents employed

* Since this discussion our attention has been drawn to the work of T Halse of direct experiments on increased fibrinolysis and thrombolysis in the presence of heparin Halse reports that while heparin is antifibrinolytic in saline or serum a marked fibrinolytic effect is observed when heparin is added to a clot in the presence of plasma This effect varies with the heparin preparation A labeled experimental thrombus was produced in rabbits by the use of dithizon thorium β complex salt and the rate of lysis of the thrombus followed by the radioactivity of the peripheral blood The injection of heparin or thromboide caused a marked elevation of activity for some hours indicating an increased rate of thrombolysis in the presence of anticoagulants (18 19)

in the treatment of thrombosis T Halse(20) has some good X ray evidence of more rapid reopening of thrombosed vessels under anticoagulant therapy He endeavors to prove that fibrinolysis *in vitro* is accelerated by the presence of heparin and the heparinoid thrombicide *His techniques are crude and we have failed to find* any effect of these agents on the several components of the fibrino lytic enzyme system of dog serum which we are now studying quantitatively by standard *in vitro* methods

H P Wright We have been trying to produce standard thrombi in animals using sodium morrhuate which of course is artificial and then dividing our animals into two groups—one group kept as controls and having no treatment and the others receiving anti coagulant dicumarol or tromexan I think we now have quite good evidence that the dicumarolized animal does get rid of its clot more rapidly than the control animals Personally I have felt that it is the liberating of the normal mechanism of fibrinolysis by the retardation of further coagulation rather than probably an active mechanism of the anticoagulant

We have been trying to find out whether the rate at which a clot disappears from its site of production is influenced by anticoagulant treatment(21) For this purpose we have produced thrombi of standard length in the *marginal ear veins of rabbits by the local* injection of 10 per cent solution of sodium morrhuate retained *in situ* for ten minutes by pressure Half of the animals received 200 mg tromexan by mouth daily from then on while the other half were kept *without treatment* as *controls Phlebograms using* 70 per cent pyelosyl as contrast medium were taken on the day following the thrombosing injection and *subsequently at weekly* intervals until the vein was clear Figure 12 shows the phlebograms of a control animal initially and at the end of six weeks *There* is virtually no change and the vein is still occluded

Figure 13 shows the phlebograms from a treated animal The initial picture is *not altogether satisfactory as there is no retrograde* filling to show up the size of the clot But they are standardized for length as nearly as we can manage by the process of production. In this treated animal the vein was patent *after two weeks* This work is still going on and we *find that the time of clearing the vein* is always *faster in the treated rabbits than in the controls* We are modifying our method of doing this work *It is* difficult to take weekly phlebograms *in* rabbits because any leakage of the pyelosyl



INITIAL 6 WEEKS LATER VEIN STILL OCCLUDED

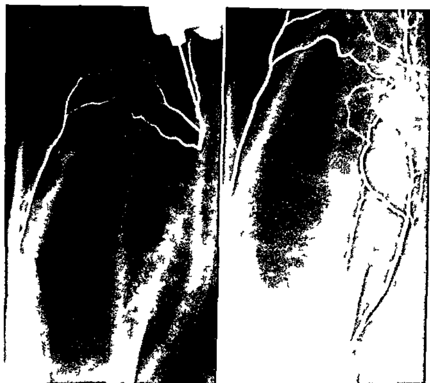
CONTROL

FIGURE 12

causes sloughing of the ear We are now introducing a small amount of barium into our morrhuate recognizing that it is a foreign sub stance In this way we can take X rays and the criticism that the foreign material may influence the absorption of the clot can be countered by having it present both in the control and in the treated animals However we are getting exactly the same thing but instead we have the clot and not the vessel showing up in the X ray

I S Wright Dr Jaques have you concluded your discussion?

Jaques There are two other points One has just been covered and that is the production of intravascular thrombosis by the injec



INITIAL 2 WEEKS LATER VEIN CLEAR

TREATED

FIGURE 13

tion of chemical agents damaging the intima The other concerns Roskam(22) who makes the point that his methods of study of hemostasis may be of significance in studying thrombosis as you know he uses a standardized bleeding time Inasmuch as Dr Barker raised the point as to which factor in a given patient is the one that precipitates the thrombosis I think Roskam s experiments on hemostasis are of significance emphasizing the fact that various mechanisms e g platelets vasoconstriction etc are acting and that probably not one single factor is the sole precipitating element In general the results obtained by these various approaches support Virchow s original ideas that thrombosis is a result of or involves a) changes in the constitution of the blood b) damage to the blood vessels and c) disturbance in the circulation As Dr Kniselv has suggested I think that these other types of direct study of thrombosis still require further investigation because we need to

work out the mechanisms whereby these three different factors produce thrombosis Further study along this line may give us further information in this regard

I S Wright Dr Brinkhous will you start the discussion of Studies on Canine Hemophilia ?

REFERENCES

1 Clark E R and Clark E L Observations on change in blood vascular endothelium in living animal *Am J Anat* 57 *385 (1935)*

2 Knisely M H *et al* *Selective Phagocytosis I* Copenhagen Munksgaard 1948

3 Peck H M and Hoerr N L The intermediary circulation in the red pulp of the mouse spleen *Anat Record* 109 *447 (1951)*

4 ——— The effect of environmental temperature changes on the circulation of the mouse spleen *Anat Record* 109 *479 (1951)*

5 Warner L Unpublished thesis Bio Medical Library University of Chicago

6 Knisely M H *et al* Microscopic observations of the circulating blood of nine healthy normal horses all of which had unagglutinated circulating blood cells and high *in vitro* erythrocyte sedimentation rates *Am J Med Sc* 219 *249 (1950)*

7 Knisely M H Eliot T S and Bloch E H Sludged blood in traumatic shock microscopic observations of precipitation and agglutination of blood flowing through vessels in crushed tissues *Arch Surg* 51 *220 (1945)*

8 Odell L D Aragon G T and Pottinger R E Relationship between erythrocyte sedimentation rate sludged blood and plasma proteins during pregnancy *Am J Obst & Gynec* 54 *596 (1947)*

9 Zilliacus H *et al* (References listed in An Annotated Bibliography on Sludged Blood to be published by *Postgraduate Medicine* in 1951)

10 Ferguson J H Observations on living mesenteric capillaries *J Lab & Clin Med* 22 *462 (1937)*

11 Laufman H Martin W B and Tanturi C A Effect of heparin and dicoumarol on sludge formation *Science* 108 *283 (1948)*

12 Heimbecker R O and Bigelow W G Intravascular agglutination of erythrocytes (sludged blood) and traumatic shock *Surgery* 28 *461 (1950)*

13 Galen C Black Bile *Corpus medicorum Graecorum Quae Extant* V 106 Leipzig Cnobloch 1823

14 WOOLDRIDGE L C *On the Chemistry of the Blood and Other Scientific Papers* London Kegan Paul Trench Trübner & Co 1893

15 MASON E C Blood Coagulations The production and prevention of experimental thrombosis and pulmonary embolism *Surg Gynec & Obst* 39 *421 (1924)*

16 WELCH W H *Papers and Addresses*, Vol I Baltimore Johns Hopkins Press 1920

17 RABINOVITCH J and PINES B The effect of heparin on experimentally produced venous thrombosis *Surgery 14 669 (1943)*

18 HALSE T Zur Wirkung des Heparins auf die Fibrinolyse *Enzymologia* 13, *176 (1949)*

19 HALSE T PHILIPP K and RUF F Tierexperimentelle Untersuchung uber intravasale Thrombolyse mit Heparin und Thrombocid *Langenbecks Arch u Dtsch Z Chir* 263 459 *(1950)*

20 HALSE T Heparin und Heparinoide Dicumarol Moglichkeiten und Ergebnisse einer thrombostatischen und thrombolytischen Therapie Stuttgart and Zurich S Hirzel 1950 (p 225)

21 WRIGHT H P and KUBIK M Coumarin treatment in experimental thrombosis *Proc Internat Cong Haem*, 1950 (in press)

22 ROSKAM J and FONTAINE R *Thrombose Veineuse* Paris Masson 1950

*23 QUICK A J Coagulation defect in sweet clover disease and in hemorrhagic chick disease of dietary origin consideration of source of prothrombin *Am J Physiol* 118 *260 (1937)*

*24 CAMPBELL H A *et al* Studies on hemorrhagic sweet clover disease bioassay of hemorrhagic concentrates by following prothrombin level in plasma of rabbit blood *J Biol Chem* 138, *1 (1941)*

*25 QUICK A J Anticoagulants effective *in vivo* with special reference to heparin and dicumarol *Physiol Rev 24 297 (1944)*

*26 BIERCE A *In the Midst of Life Tales of Soldiers and Civilians* (Introduction by George Sterling) New York Modern Library 1927

*27 FERGUSON J H Observations on alterations of blood platelets as factor in coagulation of blood *Am J Physiol* 108 *670 (1934)*

* Dr Knisely has added these references which do not appear as part of this paper

STUDIES ON CANINE HEMOPHILIA*

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HEMOPHILIA IS A disease that has commanded an enormous interest over the past several decades both because of its inheritance and clinical manifestations and because of the nature of the clotting defect Interest in the disease is indicated by the large number of publications dealing with hemophilia A cursory check of the literature revealed more than 1500 titles related to one phase or another of this disease This figure it seems to me is quite impressive when one considers the incidence of the disease The best data on this subject appear to be those collected in the Scandinavian countries In Denmark in 1943 Andreassen(1) found 81 hemophiliacs in a male population of 1 82 million The incidence would be about 4 5 hemophiliacs per 100 000 live males Since his data suggested that hemophiliacs lived only about one third to one fourth as long as normal males the frequency appears to be roughly about 13 5 to 18 0 hemophiliacs per 100 000 live male births Similar data collected by Skold(2) in Sweden revealed 101 hemophiliacs among about 3 28 million males These data suggest a slightly lower incidence of about 3 1 per 100 000 live Swedish males or about 9 3 to 12 3 per 100 000 live births If the incidence in the U S A is comparable to that in the Scandinavian countries a minimum of 2300 to 3300 overt cases of this disease exist This calculation is based on the projected census for July 1949 which indicated there were 73 76 million males in the U S A There are to our knowledge no good data to indicate the ratio of mild relatively asymptomatic cases to obvious easily diagnosed cases of hemophilia so that it is not possible to speculate on the total incidence Judging from statistics on the incidence of various types of cancer it appears that there are as many cases of hemophilia at any one time as there are cases of carcinoma of the tongue or of the esophagus or perhaps even as many cases as of brain tumor

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In starting this discussion we will not deal with human hemophilia but with an apparently identical disease in dogs which we have been studying for several years The nature of the disease in this interesting strain of dogs was recognized originally by Field Rickard and Hutt(3) Their diagnosis of hemophilia was based on the hemorrhagic phenomena the prolonged clotting time and an occurrence limited to the male offspring of certain females We obtained the female transmitters of this disease early in 1947 and have been studying their descendants since that time Clinically the hemorrhagic tendency is manifest very much as it is in humans The commonest manifestation is a chronic recurring hemarthrosis involving particularly the large joints Unless controlled by transfusions with normal blood or plasma the animals practically always succumb to massive hemorrhage during the first few months of life(4) We have made many types of studies of these animals including morphologic studies particularly on the joints but this afternoon only our data on the inheritance of the disease and some of our findings regarding the clotting defect will be presented

GERM CELLS	$X\underline{X}$		XY	
ZYGOTES	XX^{+}	$\underline{X}X^{+}$	XY	$\underline{X}Y$
NUMBER	9	7	39	36
% (ACTUAL)	56	44	48	52
% (EXPECTED)	50	50	50	50

DATA FROM
15 Litters 145 Progeny
88 Males 75 Tested
59 Females 36 Tested (Clotting)
16 Tested (Breeding)

+ 36 Females Non-bleeders

Figure 14

First a summary of our genetic studies will be given Like human hemophilia the disease is inherited as a recessive sex linked characteristic Figure 14 shows the results of our first type of cross in which transmitter females (XX) were mated with normal males (XY) It will be seen that the actual distribution into the four genotypes — normal females transmitter females normal males and hemophilic males — is very close to the expected

To carry these studies further it was necessary to rear the bleeder males to maturity This was accomplished by careful observation for hemorrhagic phenomena and prompt treatment with plasma transfusions In 1948 and 1949 it was possible to mate these bleeder males with both normal and transmitter females Figure 15 summarizes our limited data obtained from the mating with normal females Here again the results are in accord with what was expected The testing of the females for heterozygosity is incomplete To date only one of these animals has been mated She proved to be a transmitter as judged by the presence of hemophilic pups in her offspring

GERM CELLS	XX	X̲Y
ZYGOTES	XX̲	XY
NUMBER	(17+)	13
% (ACTUAL)	(100+)	100
% (EXPECTED)	100	100

DATA FROM 4 Litters 37 Progeny
18 Males 13 Tested
19 Females 17 Tested for Clotting Defect

+17 Non-bleeders 1 Transmitter Proven by Breeding

FIGURE 15

The other cross with the hemophilic male is the one with the transmitter female(5) From this mating female bleeders should result since both gametes may furnish an affected X chromosome It was with much interest that we awaited the outcome since there is such a voluminous and contradictory literature regarding the occurrence or rather nonoccurrence of female hemophiliacs in humans The results are shown in Figure 16 Here again the actual and expected findings are extremely close

With the demonstration that canine hemophilia may occur in the female we have concentrated our attention on the disease in this sex Clinically the hemorrhagic tendency is as severe as in the male In rut there is a moderate excess in bleeding but in no case has this been fatal Thus far only a few of the animals have reached sexual maturity Of those that are mature we have been relatively unsuccessful in breeding experiments From seven attempts at breeding either naturally or by artificial insemination we have succeeded in obtaining only two litters During these attempts two of the animals died of massive hemorrhage in one hemorrhage appeared due to the trauma of coitus and in the other

GERM CELLS XX XY

ZYGOTES	XX	XX	XY	XY
NUMBER	19	(20*)	21	25
% (ACTUAL)	49	(51*)	46	54
% (EXPECTED)	50	50	50	50

DATA FROM
14 Litters, 120 Progeny
60 Males, 46 Tested
60 Females 39 Tested

+ Non-bleeders

Figure 16

massive intra uterine hemorrhage occurred in the eighth week of gestation Figure 17 shows the results of the two successful breedings All of the progeny regardless of sex were bleeders There remains the cross between the hemophilic female and the normal male which we have not yet attempted

GERM CELLS	XX	XY
ZYGOTES	XX	XY
NUMBER	5	9
% (ACTUAL)	100	100
% (EXPECTED)	100	100
DATA FROM	2 Litters 14 Progeny All Tested	

FIGURE 17

Before leaving our genetic studies the difficulty in detecting transmitters by breeding should be pointed out It has been the hope of most investigators that a practical laboratory test could be devised for this purpose Hopeful reports have appeared frequently two of them in 1950(6 7) We have applied a number of tests to the female heterozygotes but to date have been unsuccessful in detecting any difference from normal females Table III shows the results of two stage prothrombin consumption tests in one set of experiments The prothrombin disappeared at about the same rate in normal and transmitter animals In other experiments no significant differences between these dogs were obtained with the one stage prothrombin consumption test A prevalent idea expressed by geneticists is that there is one gene for each enzyme

A quantitative relationship between the number of genes per chromosomal pair and the amount of enzyme produced might exist

TABLE III

Prothrombin Utilization Rates
Normal and Heterozygous Females
(Average 3 Paired Expts)

Time Min	Residual Prothrombin Per Cent		Clotting Time Min	
	Normal	Heterozygote	Normal	Heterozygote
0	100	100	7	6 5
10	76	78	–	–
20	25	30	–	–
30	6	<5	–	–

Thus, one might expect that the clotting capacity of the blood of *heterozygous females would be intermediate between that of normal* and hemophilic females If true we have not succeeded in detecting this difference Only by breeding tests have we been able to recog nize the heterozygotes This procedure has proved to be both expen sive and time consuming On an average fifteen months are re quired to test each female for the carrier status For some animals nearly two years are required since when mated with a normal all of the male offspring may be normal even though the dam is a transmitter From twenty nine matings of known female heterozy gotes we have had four litters in which none of the males were hemo philiacs This occurred in two litters in succession from one female There were six males in the first litter five in the second For practical purposes we have considered a female as normal if in two successive litters having ten normal males there were no bleeders According to probabilities of Mendelian ratios based on expansion of the binomial five normals in one litter would give a P value of 0 03 that the mother was a transmitter Fortunately this breed of dogs has large litters with a predominance of males Also we have been able to use hemophilic sires recently so that one can consider both male and female progeny in calculating probabilities This eases the difficulty of determination of female heterozygosity somewhat

Only part of our studies on the clotting anomaly in these animals will be summarized All of the reported characteristics of human

hemophilic blood which we have tested in these animals have been found to exist The *clotting time* (modified Lee White method) is prolonged to about thirty minutes to several hours A rough correlation appears to exist between the severity of the disease and the delay in clotting Hemophilic pups during the first week of life have shown the longest clotting time some bloods have not clotted overnight A deficiency in other clotting factors in the newborn particularly prothrombin may have influenced the results The *bleeding time* is normal unless the puncture is made in tough fibro cartilaginous tissues of the ear or unless large incisions are made In the latter cases active bleeding stops in two to four minutes as a rule bleeding starts again after a variable period usually about an hour and continues until corrective steps as local application of thrombin or normal plasma transfusions are taken This cycle of bleeding stoppage and bleeding suggests that the element of vascular contraction in hemostasis is normally operative in these animals

A fairly systematic study of the various clotting factors has been started Although not yet complete a brief summary can be given of some of the findings(4) Fibrinogen and calcium are present in normal amounts Fibrinogen disappears slowly from clotting blood so that several crops of fibrin can be removed over several hours but on addition of thrombin to hemophilic plasma clotting occurs as rapidly as in normal preparations Prothrombin as determined by either the original or modified two stage method is also normal Both the ordinary one stage prothrombin time and the modified procedure using $BaSO_4$ treated plasma as a diluent give normal values Plasma Ac globulin appears to be present in adequate amounts A preparation furnished by Dr Seegers failed to correct the delayed clotting Also hemophilic plasma was as effective as normal plasma in accelerating the conversion of purified prothrombin to thrombin The prolonged one stage clotting time of aged oxalated plasma is corrected equally well by addition of either hemophilic or normal plasma suggesting a normal labile factor in these animals Hemophilic serum promptly develops an accelerator of the prothrombin thrombin reaction This is shown by the supernormal serum prothrombin values with the modified one stage method and by the shortened activation period in the two stage procedure(8) In these respects the clotting hemophilic plasma behaves like the normal control plasma Also test for serum accelerator reveals no difference between normal and hemophilic serums There are conflicting data regarding serum accelerator activity in human

hemophilia perhaps because of methods used but our findings appear clear cut Excess inhibitors of the antithrombin and heparin type have not been demonstrated Platelets are present in normal numbers and appear normally reactive in accelerating the clotting of platelet poor normal plasma

Of the positive findings in addition to delayed clotting time prolonged bleeding from large wounds and slow loss of fibrinogen from clotting plasma there is a slow evolution of thrombin and a very slow loss of prothrombin from clotting plasma and serum Normally the curve showing loss of prothrombin during clotting is S shaped while in hemophilia the curve shows little change in slope in the course of many hours Trace amounts of tissue thromboplastin will restore clotting to normal as judged by our prothrombin utilization test One third of a milligram of beef lung thromboplastin was found adequate per 100 ml. of hemophilic blood (about 3 gamma per milliliter) This incidentally is similar to results which one of us obtained previously in human hemophilia(9)

We have been particularly interested in the factor in normal plasma (antihemophilic factor) which added either *in vivo* or *in vitro* has a marked corrective influence on the clotting of hemophilic blood A previous report to this group(10) indicated that the presence of formed elements particularly platelets is required for this corrective influence to be manifest We have followed the effects of normal plasma on the clotting of hemophilic blood mainly by our two-stage prothrombin consumption test Less than $1 \times 10^{-}$ ml normal plasma per ml hemophilic blood will cause a perceptible change in clotting Figure 18 shows the result of a transfusion of normal plasma to a hemophilic dog It will be noted that the greatest effect on prothrombin utilization was immediately after transfusion Even then the clotting defect was not corrected completely To give a normal rate of prothrombin consumption it appears that more than 10 per cent of the circulating plasma in the hemophilic animal must be normal

Alexander Does this correlate well with the *in vitro* additions?

Brinkhous Our data have not been analyzed statistically for correlation However the general impression obtained from inspection is that a good correlation exists

It would appear then that we are dealing with a plasma factor which is effective in traces, but which is needed in relatively large amounts to correct completely the slow clotting of hemophilic

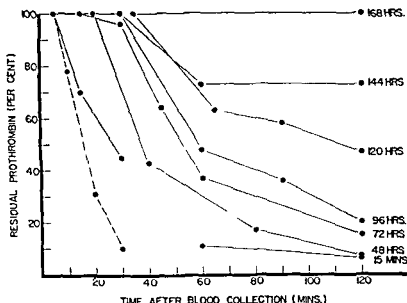


FIGURE 18 A chart showing the effect of a fresh normal citrated plasma transfusion on prothrombin utilization in a hemophilic dog The dose of plasma was 2 7 ml per kilo of body weight The solid lines indicate the prothrombin conversion rate at intervals after transfusion The interval is indicated on each curve The broken line indicates the conversion rate of whole blood of the donor animal Reprinted by permission from *J Exp Med 90 107 (1949)*

blood The range from minimal to maximal effective dose is more than a hundred thousand fold

All of our studies are compatible with the general idea that the block in clotting in hemophilic blood is due to a plasma abnormality resulting in defective thromboplastic activation of prothrombin The basis for the ineffective thromboplastic activity has been a subject of debate We are all aware of the divergent ideas on the subject which in general fall into three categories a) there is a deficiency in a procoagulant either a plasma thromboplastin as Howell and others have suggested or a plasma factor concerned with the mobilization formation or action of thromboplastin b) there is an excess of an anticoagulant and c) there are both a procoagulant deficit and an anticoagulant excess Our opinion is that in hemophilia there is a deficiency in a plasma procoagulant not itself active thromboplastin which requires platelets to make thromboplastin available for clotting Although we have made suggestions previously as to the nature of this reaction the body of data required to reach a definitive conclusion is still inadequate

The last studies which we wish to report deal mainly with our attempts to assay the corrective factor in normal plasma In all assay procedures whole hemophilic blood or platelet rich plasma is mixed with normal plasma and the acceleration in clotting observed

The oldest attempts to assay the corrective factor in normal plasma were made over a decade ago using the reduction in clotting time of hemophilic blood after addition of normal blood or plasma Several modifications of this procedure exist We have used this approach but have not succeeded in obtaining duplicable results The end point is difficult to determine accurately especially in slowly clotting bloods In addition the clotting times observed correlated very poorly with other indices of the clotting rate

A second method for determining the rate of clotting which we have used employs the comparatively recently devised procedure the one stage serum prothrombin" time With clotting normal blood there is first a period of prothrombin" hyperactivity followed by a decline over a very few hours With clotting hemophilic blood the period of hyperactivity develops as in normal blood but the subsequent decline is very slow (8) When normal plasma is added to hemophilic blood intermediate type curves are obtained While better results are obtained than with the clotting time method much unexplained variability occurs

The basis for the third type of assay of antihemophilic activity and the one with which we have had the most experience is the determination of residual prothrombin by the two stage procedure in whole hemophilic blood Basically the procedure is the same type as described in 1939(9) Simultaneously dilutions of plasma or other material being tested for antihemophilic activity are mixed with a standard volume of whole hemophilic blood After varying periods of time the residual prothrombin is determined The greater the concentration of antihemophilic factor in the mixture the more rapid the loss of prothrombin from the hemophilic blood By using a platelet poor hemophilic plasma instead of whole hemophilic blood possible errors due to contaminating tissue thromboplastin can be detected This general approach to the assay of antihemophilic factor has been used in our laboratory for several years during which time several modifications in the actual procedure have been made These modifications have to do a) with the amount of test material added to hemophilic blood and b) with

the reaction time Examples are given below to illustrate the results obtained with three separate modifications of this assay procedure

Figure 19 shows the results obtained in one experiment in which the antihemophilic activity of normal dog serum and human Fraction I was compared with normal dog plasma In doing these assays serial dilutions of plasma serum and solutions of Fraction I were made Then a standard volume of each dilution was mixed with 1 ml whole hemophilic blood At intervals the residual prothrombin was determined For one assay some thirty to forty two-stage prothrombin determinations are required In the figure only those results are plotted which permit ready comparisons between the materials being tested Inspection of these data indicate that the serum and the Fraction I contain only about 1 per cent as much antihemophilic activity as does the normal plasma This procedure is too laborious for general use even in a research laboratory

Figure 20 shows the results of repeated determinations of antihemophilic activity in clotting dog plasma using a somewhat simplified assay procedure Only one concentration of the test material (1 to 10 dilution) was added to the whole hemophilic blood and the reaction time was kept constant (thirty minutes) A single determination of residual prothrombin in the whole hemo-

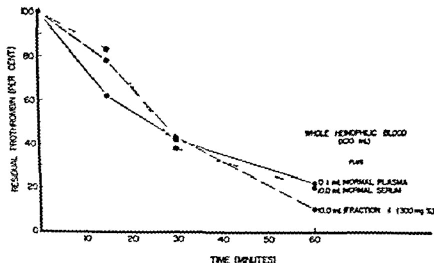


Figure 19

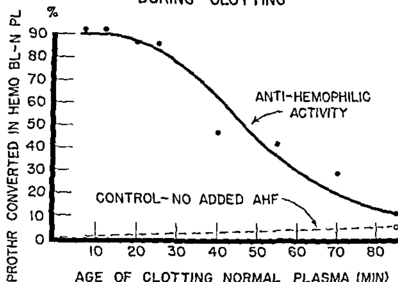


FIGURE 20 Native dog plasma (52 000 platelets per cmm) was prepared by centrifugation using siliconed equipment at 40 C At zero time the normal plasma was transferred to plain glass tubes at 28 C and allowed to clot At intervals a sample was added to hemophilic blood freshly obtained for each test and incubated thirty minutes at 28 C Saline was mixed with hemophilic blood in the control.

philic blood then furnished an index of antihemophilic activity It will be noted that during the course of clotting the normal plasma (or serum) gradually lost its ability to cause conversion of prothrombin in the hemophilic blood It should be pointed out that here we have another index of the rate of clotting This is a test for *consumption or utilization of antihemophilic factor* during clotting This utilization test is adaptable to the study of a number of clotting abnormalities It cannot be used of course in instances in which the plasma is largely lacking in antihemophilic activity just as the prothrombin utilization test is of limited value in severe hypoprothrombinemia We have used this procedure in the study of the clotting of thrombocytopenic plasmas in which the platelet levels had been varied by regulated centrifugation With reduced platelet levels the rate of loss of antihemophilic factor during clotting is delayed

Out of the experience with the two procedures just described there has evolved our current method for determining antihemophilic activity This method permits expression of the antihemo

philic activity in per cent of normal control plasma Varied amounts of unknown and control are added to the hemophilic blood while the reaction time is kept constant Figure 21 shows the manner in which the results are finally plotted The prothrombin residual in per cent is indicated on the Y axis the volume of added material x 10^3 per milliliter hemophilic plasma on the X axis (correction was made for hematocrit values of the hemophilic blood and for the dilution of normal plasmas with anticoagulant) A comparison is made between the volumes of added materials which would result in residual prothrombins of 70 50 and 30 per cent respectively as indicated in the figure An average of the ratio (unknown/control) x 100 represents in per cent the amount of antihemophilic factor contained in the unknown in relation to the control In this case the result was 104 per cent This particular assay was done with plasma from a dog with liver damage due to prolonged inhalation of chloroform At the time the plasma was obtained the prothrombin and fibrinogen were at low levels Antihemophilic activity was normal however These data suggest that the antihemophilic factor is not formed in the liver

It will be observed that the relationship between residual prothrombin in the hemophilic blood and added plasma is roughly linear (Figure 21) On the basis of this relationship antihemophilic activity may be expressed in terms of prothrombin units lost in a given hemophilic blood under the standard conditions of this test A unit of antihemophilic factor may be established on this basis Thus one unit of antihemophilic factor could be that activity causing conversion of one unit of prothrombin under the standard conditions of the test In this case the unit would be quite small Normal dog plasma in the example given (Figure 21) would contain roughly 14 400 units per milliliter as the hemophilic plasma contained about 400 units of prothrombin per milliliter A somewhat larger unit would be desirable If one unit were that amount which under standard conditions will convert either 10 units or 100 units of prothrombin in hemophilic blood then the dog plasma would contain either 1 440 or 144 units per milliliter depending on which size unit is selected More study and experience with this procedure for measuring antihemophilic factor is needed At present we prefer to express the amount of antihemophilic activity as a percentage of a normal control plasma using blood from a single hemophilic dog for each assay

I S Wright I will ask Dr Quick to open the discussion

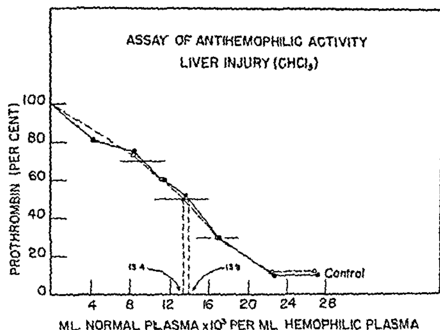


FIGURE 21

DISCUSSION

Quick I have been very much interested in Dr Brinkhous work and I think that his work on the genetics has been invaluable Certainly it has answered some questions that never before have been answered Whether all the data can be applied to human hemophilia remains for further study to determine

As you probably know in a little village of Tenna in Switzer land hemophilia had its heyday The population of Tenna in 1854 was one hundred and five and it is recorded that there were sixteen bleeders — eleven males and five females But the statement was made that these five female bleeders merely had heavy menstrua tion Therefore it leaves us somewhat in doubt as to whether they were true hemophiliacs But when we have an incidence of eleven male hemophiliacs in a population of one hundred and five it is very probable that some carriers were mated with hemophiliacs and yet there is no good evidence that a case of hemophilia in the female occurred To the best of my knowledge no case of true hemophilia in a woman is on record *

* *A report has now appeared See Israels et al Lancet 260 1365 (1951)—Editor*

There are cases of hemophilia like diseases but these differ from true hemophilia

Now a word about the incidence of hemophilia I think the figures are a little low From my own study in my own community Milwaukee I have found more than twenty hemophiliacs in a population of about 600 000 Assuming that half of the Milwaukeans are males and that at least ten more hemophiliacs have not come to my attention would indicate that there are about thirty hemo philiacs in Milwaukee — an incidence of about ten in 100 000 males

It must be stated however that some of these cases are very mild yet nevertheless can be classed as hemophiliacs on the basis of a bleeding tendency hereditary history and laboratory findings

Mention should also be made of a hemophiliac disease in pigs Curiously enough that disease is not sex linked and in order to get active bleeders you have to mate a female carrier with a male carrier Apparently you can t get an active bleeder by mating a hemophilia carrier with a normal irrespective of sex(11)

As you would expect differences of opinion arise particularly in regard to the nature of this disease Historically Alexander Schmidt recognized that the agent that was lacking in hemophilia was thromboplastin or "zymoplastic" substance In fact Manteuffel(12) demonstrated that by adding tissue extract to hemophilic blood the clotting time became normal

I think that was brought out particularly well by Sahli(13) Bancroft Stanley Brown and I(14) showed that the prothrombin as measured by the one stage method was normal in hemophilia and delayed in various types of jaundice There then arose a division as to where the defect actually was

Much effort has been spent to determine whether the hemophilic defect is in the plasma or in the platelets Bendien and Creveld(15) Patek Taylor and their associates(16 17 18) Lenggenhager(19) and others hold that the hemophilic defect is in the plasma Others considered the defect to be in the platelets Brinkhous(20) postu lated that the defect in hemophilia — I quote "In hemophilia the prothrombin conversion is extremely slow throughout the entire period and an extremely small amount of thromboplastin brings it to normal"

And in the conclusion he says "Emphasis is placed on the form elements of the blood and the sluggishness with which they liberate thromboplastin" In other words platelet furnished the thrombo- plastin which is an idea that many held at that time

In 1947 Dr Brinkhous (21) and I(22) at the Federation meetings both reported on the coagulation defect and in his results which are printed in the *Proceedings of Experimental Biology and Medicine* in 1947 Dr Brinkhous(23) concluded that normal plasma contains a factor necessary for thromboplastin liberation from the formed elements of the blood presumably from platelets

The idea is still held that the platelets furnish thromboplastin In the last article which is on canine hemophilia in the *Journal of Experimental Medicine* — this is still quoting "Recent studies point to an impaired mobilization of thromboplastin which prevents conversion of adequate amounts of prothrombin to thrombin Thus it appears that normally thromboplastin becomes available in shed blood as the result of the interaction of platelets and the corrective antihemophilic principle in plasma (4) I dont know whether Dr Brinkhous still maintains that or not It is only for a matter of discussion that I bring these things up not for arguing as in a debating society

I stress these things because human lives depend upon our concepts and our treatment and therefore I want to present to you what I consider some of the things that are worthy of discussion

In 1947 I(24) brought out the idea that the platelet does not furnish thromboplastin but furnishes an activity which reacts with an inactive precursor of thromboplastin to change this to thromboplastin

Seegers On that theory of yours how do you know which is the substrate and which is activator and what is the evidence that the platelets contain the activator?

Quick I have no conclusive evidence which is the substrate and which is the activator but both a platelet factor and a plasma factor are required for the formation of thromboplastin In thrombocytopenia as in hemophilia little conversion of prothrombin occurs When hemophilic blood and thrombocytopenic blood are mixed normal consumption of prothrombin takes place(25)

On the other hand when we add to hemophilic blood the lysed platelets (the active material that we had tested on thrombocytopenic blood and on blood from which we had removed the platelets) we get little or almost no consumption of prothrombin Therefore there are two separable factors and I think that for the time being at least we can be permitted to call that the precursor of thromboplastin

Seegers How can you if you don t have the evidence? Which one is substrate and which is the activator? You haven t any evidence have you?

Quick It is just a question of nomenclature but the important consideration is that the amount of active thromboplastin formed acts stoechiometrically with prothrombin

Seegers How do you know that?

Quick Simply because we have carried out experiments which I will put on the board in a minute

Alexander Dr Quick you said that platelets are relatively devoid of thromboplastin How do you know this?

Quick I shall first answer Dr Seegers When we add tissue thromboplastin to hemophilic blood we can increase prothrombin consumption thus suggesting this end product of the platelet and the plasma factor acts like tissue thromboplastin Now which of these is which? That cannot be answered positively but from a clinical point of view the significant fact is that there are two distinct factors which are necessary to produce thromboplastin

Seegers Well how are you going to get around that? You don t know whether the substrate is in the platelets or whether it s in the plasma and you have no evidence for it You are just assuming that you know the answer It seems to me that that is the case

Quick From the curve relating prothrombin consumption to the number of platelets(26) it was concluded that the activator resided in the platelets

I S Wright There is another question that has not been answered—one by Dr Alexander How do you define the term thromboplastin Dr Quick

Quick Thromboplastin is the agent which with an adequate amount of labile factor and calcium causes the prothrombin to be changed to thrombin

Alexander And you still reiterate and claim that platelets are devoid of the ability to convert thrombin to prothrombin?

Quick In the absence of the plasma factor platelets alone can not in the presence of labile factor and calcium convert pro thrombin to thrombin

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reduction in the number of platelets when washed human platelets have been mixed with purified thrombin

Flynn Dr Quick quoted Fonio s work where there is a statement that thrombin produced an effect on the appearance of platelets However I do not think he said it caused disintegration did he Dr Quick?

Quick A translation of Fonio s statement is as follows "We have in all our observations with native preparations seen how the addition of thrombin causes an increased formation of pseudopods assisting their attachment and further how the formation of fibrin needles suddenly fixes them and brings about their disintegration" The important question is when is the platelet disintegrated? When the platelet actually disintegrates it produces globular material which can still be centrifuged out

Seegers Well it seems to me Dr Quick that you are postulating that thrombin alters platelets and we have no definition of what you mean by altering and also that the idea for the most part is one of theory and not of fact not of experimental evidence

Edsall May I also ask a question about Fonio s work — what was the nature of the thrombin preparation he used and how high was the concentration of thrombin in his experiments?

Flynn That is a good question Actually I couldn t find in his monograph that he mentioned his source of thrombin or the degree of purity of it isn t that right Dr Quick?

Quick The article was in the *Schweizerische medizinische Wochenschrift* (28)

It probably was not the highly purified thrombin that is on the market But thrombin was produced a long time ago and it probably was a preparation that most of us had been using the type that Mellanby developed

Flynn It is correct though that he did not state what material he used

Quick To the best of my knowledge that is true

Zucker Both Fonio and Zatti produced platelet alterations by adding thrombin to platelets suspended in plasma Their results therefore do not necessarily demonstrate that thrombin itself alters platelets

Alexander Did you ever take platelets and attempt to produce thrombin from prothrombin under those conditions to see if any thrombin is evolved?

Quick In intact blood or intact plasma? The minute you introduce artificialities you get into trouble

Alexander How does one know that the minute it does not work with plasma, one gets into trouble?

Link I would like to insert here that Dr Tocantins has pointed out(27) that in the Quick prothrombin determination on "100 per cent plasma the dilution of the plasma is — what was that figure again?

Brinkhous Twenty six per cent

Link Dr Quick you are not on solid ground

Quick I will come to that question Dr Link in a moment The next thing I want to present for your consideration is the intricacies that we get into when we are talking about prothrombin consumption Not only do we have the interaction of two agents to form thromboplastin but thrombin is necessary to break up platelets in *order to make the platelet factor available This is complicated by* the fact that thrombin is being absorbed to fibrin and thus constantly taken out of the reaction mixture so that this disintegrating effect is inhibited

Alexander Does the addition of purified thrombin to a suspension of platelets cause the platelets to disappear?

Quick I would like to show you some indirect evidence

Seegers I would like also to ask whether or not you have ever mixed purified thrombin with platelets to see whether that happens Have you ever done that?

Quick Fonio(28) and more recently Zatti(29) have reported that thrombin acts on platelets bringing about their disintegration We also have *observed that thrombin affects platelets* but we did not use highly purified thrombin

Seegers Well suppose we drew the conclusion that thrombin does not cause disintegration of platelets What would you say to that?

Alexander We have tried many times to study the interaction of platelets with thrombin and we have failed to demonstrate any

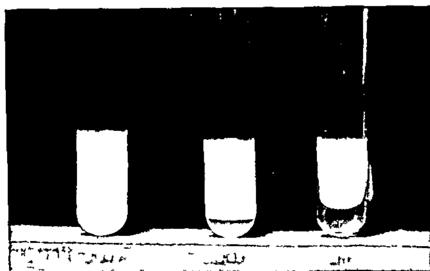


FIGURE 2. Influence of Thrombin on Clot Retraction. Washed human platelets were suspended in purified fibrinogen solution Thrombin was prepared according to the directions of Quick Reprinted by permission from Science 112 538 (1950) *and Am J Med Sc* 220 *538 (1950)*

We routinely do platelet counts by adding sodium citrate Thus there are five conditions in which the platelets are stable and in each the formation of thrombin is stopped

Flynn Surely you have another common factor there and that is delayed fibrin formation

Quick Fibrin can be ruled out because platelet agglutination readily occurs in afibrinogenemic blood unless it is citrated as Pinniger and Prunty(31) have shown

Alexander Aren t you implying Dr Quick that the only possible reactions in clotting blood are a) the deposition of fibrin and b) the conversion of prothrombin to thrombin?

Quick No I think that is far from the story

Alexander Well if it is far from the story may not the rest of the story be just what the mechanism is for platelet destruction neither the deposition of fibrin nor the conversion of prothrombin to thrombin?

Kay May I ask if you controlled your study by testing various dilutions of thrombin against fibrinogen without the addition of platelets?

Seegers I think you ought to correct the statement that you made a moment ago There is no highly purified thrombin on the market today and I don t think Fonio had very potent or very purified thrombin

And you might also correct another statement that Mellanby prepared purified thrombin That is also not correct

Quick I said the thrombin preparation that most of us have been using

Seegers I mean your statement in the literature that he prepared pure thrombin

Quick Well certainly pure is a relative term

Tocantins Back in 1917 Wright and Minot(30) studied the effect of several substances present in the blood and in serum for the property of producing the viscous transformation of platelets and one of the conclusions they reached was that purified thrombin alone did not produce those changes

Quick Much of our information must of necessity be obtained indirectly The following experiment is an example that thrombin affects platelets On adding 0 002 ml of thrombin to a 2 ml suspension of washed platelets in fibrinogen solution complete clotting but no retraction occurred When the thrombin was increased to 0 05 ml clot retraction occurred as shown in second tube and 0 1 ml of thrombin caused considerable more retraction as shown in Figure 22

Now anyone who will deny that there is a relationship of thrombin to platelet lysis is ignoring some very important facts because whenever you depress thrombin formation you stabilize platelets

To illustrate in very severe hemophilia the platelets are relatively stable similarly when you reduce the prothrombin with dicumarol to a very low level you can do a platelet count on the plasma without adding any anticoagulant because the platelets do not agglutinate When you pass blood through amberite which takes out the calcium the platelets become stable and can be counted without addition of any further anticoagulant

When heparin (purified heparin) is added to blood a platelet count can be made on that blood without any difficulty No agglutination is encountered

ducing relatively small amounts of proteolytic enzyme I am not at all sure whether these two agents have anything in common

Kay I would agree with Dr Ferguson that this has to be con sidered proteolysis or at least the effect of oxygen in converting some of this material to a soluble substance

Quick I cannot agree with that at all because I think I can recognize clot retraction when I see it

I dont believe that you can get this type of shrinkage as the result of fibrinolysin

Kay When you say fibrinolysin are you referring to the lysis of fibrin or to the activity of plasmin? I am not talking about plasmin activity That will not produce that type of a clot in a tube but an enzyme which is active in thrombin against gelatin and against fibrin will give you exactly that type of picture

Edsall Have you tested some of the extremely active thrombin preparations such as Dr Seegers has?

Kay Dr Seegers was good enough to supply us with some of his material the assay of which I do not recall

Seegers So far as I know the most potent preparation of thrombin that we have ever had is 1 400 units per milligram of dry weight

Kay The thrombin with which Dr Seegers supplied me showed proteolytic activity against gelatin as indicated by a decreasing viscosity over a two hour period

Seegers Dr Kay did you use the commercial preparation of thrombin?

Kay I have used the commercial thrombin supplied by Parke Davis Co and your preparation as well

The material that you sent me was considerably more active per weight It was very very active but it again had this specific effect on gelatin

Now this viscometric study on proteolysis is quite accurate I feel and I control it each time with a gelatin to which none of the material which is under test has been added

Under these circumstances the viscosity of the control will not change for twenty four hours However with the thrombin which you sent me and which you reported had been freed of Ac globulin

Quick You mean just adding fibrinogen and increasing—

Kay Figure 23 looks exactly like that It shows the use of *fibrinogen and thrombin in varying dilutions The greater the* thrombin concentration the more clot retraction

It occurred to me that since the size of the final clot was a function of the amount of thrombin proteolysis might have occurred When the thrombin preparation was tested against gelatin in a viscometer proteolytic activity was indicated by decreasing viscosity over a period of two hours Thereafter the viscosity remained stable The control gelatin did not change viscosity over a twenty four hour period

Furthermore the enzyme is active against gelatin and fibrin in the presence of 5 63 x 10^5 gm per ml of ionized zinc which indicates according to my work that it cannot be plasmin since plasmin is not active in that concentration of zinc(32)

Quick You believe that this is a fibrinolysin effect?

Kay I am not sure that this represents lysis of fibrin However with the thrombin which I use I was able to get the same degree of what you call clot retraction of fibrin (Produced from Armour's Fraction I of bovine plasma) and with the same thrombin I was able to show digestion of gelatin but not of egg white

Ferguson Clot retraction does not occur in platelet free plasma clots nor in purified thrombin fibrinogen mixtures The clots in both these cases can be made to retract either by adding intact platelets (which we prepare by the silicone technique) or by intro



FIGURE 23 Thrombin concentrations in units from left to right 500 250 125 62 5 31 25 15 75 7 875 3 44 1 77 0 0885

and that the clotting activity and dissolving activity were destroyed to the same extent by heat

Alexander I gather from what has been said that there is no strict correlation between the specific thrombin activity and the specific activity or the ability of the solid to dissolve fibrin

Kay That is true but one possibility remains that the enzyme is lost fairly quickly after it is put into solution and it is conceivable that the thrombin loss proceeds at approximately the same rate

In other words I haven t been able to assay the thrombin loss in periods of time with regard to the enzyme loss The enzyme is completely gone in two hours from all preparations that I have used

Ferguson Attempts to separate the two in this way may be mis leading We relied upon parallel loss of fibrinolytic potency and ability of certain plasma-enzyme preparations to assist in blood clotting (particularly in the conversion of prothrombin to throm bin) But this evidence had to be set aside when we turned to another method namely the addition of an enzyme inhibitor (e g soybean antiprotease pancreatic trypsin inhibitor navy bean in hibitor or serum antifibrinolysin) These abolished fibrinolysis but not the clot aiding effects

Kay Neither the antiplasmin from soybean or that from bovine serum inhibits the activity of this enzyme It is inhibited by alpha tocopherol phosphate but this inhibits thrombin as well

Ferguson They will not inhibit thrombin but soybean inhibitor is antithromboplastic

Quick Dr Seegers I believe has shown that the fibrinolysin in thrombin is present in very small amounts Isn t that right Dr Seegers?

Seegers No I have not shown that I have shown that in our preparations of thrombin there is very little fibrinolysin present — practically none

I don t know what you have in your thrombin preparation but I suspect that it may be contaminated with fibrinolysin

I am not trying to suggest that clot retraction is due to fibrinolysis As far as the thrombin preparation you used you really have no information on it at all as far as I can tell You only know that probably you had about 50 units per milligram and you have no

by Dr Ware the viscosity changed over a two hour period After two hours there was no further decrease

Seegers Of course we know that if you add thrombin to fibrinogen you can obtain a clot and if you have the thrombin strong enough the clot will eventually dissolve That is when you get into a range where you are matching weight for weight

Kay I am not talking about a physiological range However with the commercial thrombin which I believe Dr Quick used you can obtain the same type of reactions with very little thrombin and the enzyme is there in considerable excess of what I found in your particular purified thrombin

Flynn What was your source of thrombin Dr Quick?

Quick Thrombin that I have prepared myself

Flynn Is there plasma in your preparation?

Quick The thrombin contains no plasma The preparation of thrombin which was employed was of such strength that when 0 1 ml was added to 0 2 ml of fresh human plasma a clot formed in three seconds

Edsall How much protein is there in 1 ml?

Quick That I can t tell you

Laki Did you say that purified thrombin had this effect?

Kay Yes the purified thrombin showed proteolytic activity against gelatin for two hours

Laki Do you consider a contamination in the thrombin preparation doing it?

Kay I cannot say that this enzyme is a contaminant I have classified it as to optimum pH which is 4 7 It is active apparently against fibrin and gelatin but it is not active against egg white when the technique described by Dr Schales is used(33) This suggests that it may be either a chymotrypsin like enzyme or perhaps a weak phosphatase — I am not sure

Laki Or thrombin itself

Kay It might be a contaminant

Laki Last year Guest and Ware(34) presented experiments showing that large amounts of thrombin slowly dissolved the clot

by modifications that lower its accuracy and destroy its clinical value

One must further consider the evidence that in the human not all the prothrombin is in the free state but that part is in a pre cursor form Bordet(35) was the first to postulate that prothrombin existed in a precursor form proserozyme The present concept differs only in that it postulates that both free and inactive pro thrombin are present in human blood The one stage method determines only free prothrombin

Seegers How do you know that prothrombin is in the precursor state? What is the evidence? I have never isolated any prothrombin that was in the precursor state and I have been trying to find some for a long time

Quick You don t get it by going to the slaughterhouse and col lecting blood in tin cans It requires keeping blood from contact with wettable surfaces Blood must be collected with utmost pre cautions in silicone coated containers the prothrombin adsorbed with tricalcium phosphate —

Seegers Are you adsorbing prothrombin or prothrombin fibrinogen?

Quick You adsorb both and on eluting it with sodium citrate you will find that if the material is kept in a silicone coated test tube that the prothrombin will stay at twelve seconds and if it is kept in glass it will go to eight seconds

Now that has been a great puzzle to us I think we have the answer in the fact that in contact with glass this material is con verted to free prothrombin

Alexander How do you know that an accelerator hasn t formed Dr Quick?

Quick You have to accept that your accelerator is adsorbed by calcium phosphate in the same way

Alexander I accept that

Quick You also have to accept that when you store plasma oxalated plasma in glass that this accelerator forms

Alexander Not only do we accept it we publish accordingly

Quick Why do you have to call it accelerator when this explana tion has just as much support?

idea how much protein there was in it I am surprised that we can even call it thrombin it is a solution containing thrombin with who knows what else there is in it

It seems to me that that is an experiment which alone doesn t *prove anything It merely suggests that probably your interpreta* tion could be correct or some other interpretation could be placed upon it

I think we started in this direction when we were asking you whether or not thrombin caused disintegration of platelets and what evidence you have for it and as nearly as I can tell it is just simply guessing or hypothecating or theorizing

And then you made another statement a little while ago that fibrin has nothing to do with it and even before that you said that the fibrin absorbs the thrombin

Now does the fibrin have something to do with it or does it not?

Quick Fibrin by adsorbing thrombin does not allow the chain reaction to precede i e the removal of thrombin prevents the lysis of platelets –

Seegers Well now one more thing How do you know that is a chain reaction?

Quick For two reasons first in the plasma of an afibrinogenemic patient the activation of prothrombin as measured by the pro thrombin consumption test proceeds very rapidly Second in marked contrast when normal blood with a normal concentration of fibrinogen clots very little prothrombin is consumed as long as clot retraction does not occur Thus if blood is allowed to clot in a test tube and is incubated for fifteen minutes or more after a solid clot has formed and then covered with a layer of sodium citrate to stop the clotting reaction one finds after centrifuging that the serum contains about as much prothrombin as did the blood If no sodium citrate is added a rapid consumption of pro thrombin occurs as soon as the blood is centrifuged because when the intimate contact of serum with fibrin is broken the removal of thrombin by fibrin is no longer efficient and the chain reaction is set off I emphasize this because it is important in carrying out the prothrombin consumption test I expect this procedure to become an important test in the clinical laboratory and I am going to do my utmost to see that it is carried out properly and not be spoiled

TABLE IV

	TIME AFTER SOLID CLOT HAS FORMED			
	15 min	30 min	45 min	60 min
	Prothrombin time of serum			
Tube 1	7	8	8	8
Tube 2		8	8	8½
Tube 3				8½

A similar pattern is obtained if the test is done on normal deplateletized plasma

Edsall Thus deplateletized plasma is from a normal individual?

Quick Yes deplateletized normal plasma In fact we employ the prothrombin consumption time of deplateletized plasma as the control blank It is necessary to know that the prothrombin concentration is normal and this is most satisfactorily ascertained from the prothrombin time of serum obtained from deplateletized plasma

In mild hemophiliacs the prothrombin consumption time is greater than eight seconds Tentatively we consider the hemophilic range to be eight to twelve or probably fourteen seconds

While the severity of the bleeding tendency in general runs parallel with the degree of deficiency of the plasma factor as measured by the prothrombin consumption test exceptions occur From the data we have so far collected it appears that the basic defect remains constant but the bleeding tendency varies It is likely that the hemorrhagic phase may be partly attributed in certain instances to a vascular disturbance superimposed on the basic defect

One remark about hereditary bleeding diseases They all appear to be due to a deficiency – a lack of an essential factor In hemophilia it is a factor in the plasma Hereditary hypoprothrombinemia is another established entity and so is lack of labile factor (Owren s parahemophilia) Afibrinogenemia is still another type of congenital deficiency

If I may have one more minute I would like to bring up the so called no man s land in the one stage method

Alexander Because of the fact that in the two stage method there is no change in the number of thrombin units that you can evolve from that fraction You cannot make more thrombin out of that fraction by the two stage method than you could originally But by the one stage method it goes faster

Seegers It seems to me Dr Quick that you have made some observations in regard to prothrombin time and like Bordeau have interpreted them to mean that prothrombin is a precursor but that you have no evidence that prothrombin is there in a precursor stage The evidence of these experiments still need to be interpreted and your interpretation is only one of dozens of other possibilities

Quick It is a hypothesis

Link What is the use of it?

Flynn Dr Quick with regard to this point of active and inactive prothrombin did not Stefanini point out there were certain facts inconsistent with the hypothesis namely that the so called depro thrombinized plasma with tricalcium phosphate which should re move both active and inactive prothrombin still had an accelerating effect on the one stage prothrombin test

Quick That is work that Stefanini did after he left my laboratory and I let him interpret his own findings

Flynn But his observations are I think not to be ignored particularly as they are inconsistent with the hypothesis

Quick Well be that as it may I want to get to the prothrombin consumption test from the point of view of clinical application

The reason that I present the theoretical aspects of the pro thrombin consumption test is because this method offers a means for studying hemophilia quantitatively In severe hemophilia due to lack of plasma factor the agent I call thromboplastinogen almost no thromboplastin is formed therefore little prothrombin is *consumed* In the test 2 ml of blood are transferred to each of three test tubes The blood is allowed to clot and after fifteen minutes the first tube is centrifuged after thirty minutes the second and after one hour the third tube The prothrombin of the serum is determined every fifteen minutes during the hour In a severe hemophiliac one obtains the pattern such as the following

Tocantins In other words a point was reached where the clotting time began to rise?

Quick For human plasma 75 mg becomes the minimum Concentrations greater then this do not shorten the prothrombin time but below 75 mg the prothrombin time becomes prolonged

Tocantins Yet when 200 mg of brain powder (which you consider "adequate") were used to prepare the thromboplastin a twelve second clotting time was obtained on the native plasma whether 0 1 ml or 0 2 to 0 5 ml of plasma were used or in other words when two three four and five times more plasma (and prothrombin) existed in the mixture The one stage "prothrombin time" therefore can hardly be said to vary indirectly as the prothrombin concentration even when an adequate" amount of thromboplastin is present in the mixture

Quick I fear I am confusing you The prothrombin time goes up when I do not use a high enough concentration of thromboplastin If instead of using an extract of 200 mg of thromboplastin in 5 ml I use 75 mg in 5 ml I obtain the results shown in Table VII

TABLE VII

Volume of native human plasma	0 1	0 2	0 3	0 4	0 5
Volume of saline	0 1				
Volume of thromboplastin*	0 1	0 1	0 1	0 1	0 1
Clotting time in seconds	12	12	14	15$_a$	17

*75 mg in 5 ml saline

Kay Are you sure that is all thromboplastin that you are using?

Quick Oh no But rabbit brain thromboplastin to the best of my knowledge contains no interfering inhibitors and no accelerators

Kay There is inositol phosphatide

Flynn Both Tocantins and Overman make their inhibitor from Quick s thromboplastin

Tocantins Yes plenty of the lipid inhibitor may be extracted from acetone dried rabbit brain

You remember that Dr Tocantins(27) pointed out that the plasma in the one stage method is diluted to 26 per cent and he presented a chart to show that when the plasma concentration is increased the clotting time becomes prolonged Is that correct Dr Tocantins?

Tocantins Yes

Quick We have carried out a similar experiment except that we employed native plasma and added thromboplastin (extract of acetone treated rabbit brain) with the following results

TABLE V

Volume of native human plasma	0.1	0.2	0.3	0.4	0.5
Volume of saline	0.1	0.0	0.0	0.0	0.0
Volume of thromboplastin*	0.1	0.1	0.1	0.1	0.1
Clotting time in seconds	12	12	12	12	12

* 200 mg of dehydrated rabbit brain in 5 cc saline

Tocantins Do I understand you correctly in saying that you used normal native plasma?

Quick Normal native plasma and hemophilic plasma Both gave a prothrombin time of twelve seconds as the concentration of plasma was increased until the thromboplastin was no longer adequate

Tocantins When was the thromboplastin no longer adequate? What is meant by that statement?

Quick If a series of thromboplastin extracts are prepared using decreasing amounts of thromboplastin and if these are tested using the one stage procedure results as the following are obtained

TABLE VI

Thromboplastin mg * †	250	200	150	100	75	50	25	10
Prothrombin time in sec	12	12	12	12	12	14	16	20

* Amount extracted with 5 cc saline
† Fresh oxalated human plasma 0.1 ml
Thromboplastin extract 0.1 ml
CaCl 0.01 M 0.1 ml

the age of thirty and resemble in most respects the case reported by Fantl and Nance(36) The clotting time of their venous blood is over three hours (silicone tubes) The clotting of the blood and plasma of these patients is accelerated by dilution and behaves in all respects like hemophilic blood The blood and plasma of these patients when subjected concurrently with the blood of hereditary hemophiliacs to most of the tests to measure the various clotting activities respond in all respects like hemophilic blood or plasma and do not do anything that hemophilic blood or plasma fail to do Like most severe hemophiliacs these patients respond in a limited degree to blood transfusions the hemostatic effect of a transfusion of normal blood is slight and transient Curiously the hemophilic women have most of the time little trouble with excessive bleeding during menstrual periods None of the three women had had blood or plasma transfusions previous to developing the hemorrhagic disease

It is important in identifying the hemophilic defect to make sure that no other defects exist in the blood that might account for the delayed coagulation There should be no excessive amount of anti thrombin or fibrinolysin activity The amount of prothrombin Ac globulin and fibrinogen the number and morphology of the platelets should be normal There should be a delay in the venous clotting time when performed in glass tubes sometimes and always when the blood is placed in silicone tubes The prolonged clotting times of venous blood and of plasma should be markedly shortened by dilution with physiological salt solution The conversion of pro thrombin to thrombin should be delayed especially when platelets or cephalin are used as activating agents The response of the plasma to an excess of a thromboplastic extract of brain tissue should be slower than normal particularly when the testing mix ture has a plasma concentration of 70 per cent or higher The slower than normal response of the plasma to platelets thrombo plastin or cephalin should be corrected by dilution of the plasma The bleeding time of shallow cuts of the skin should be normal but that of deep (5 mm or greater) cuts should be prolonged Platelet poor hemophilic plasma should delay the rate of clotting of plate let poor normal plasma in silicone coated tubes and sometimes even in glass tubes Protamine should not shorten significantly the clotting time of the blood or plasma instead it should prolong it

I should like to put several questions to Dr Brinkhous before we go on with our discussion How many of these hemophilic dogs

Quick But you started manipulating and the minute you manipulate you have something entirely different

Kay And inositol phosphatide is an inhibitor whether manipulated or not manipulated furthermore it is present in brain tissue

Quick I shall be glad to get information on whether thromboplastin prepared from rabbit brain with acetone dehydration contains inhibitors

Kay I have that information here if you would like to see it right now

Tocantins I should like to state first how much I enjoyed hearing Dr Brinkhous discussion His work on the hereditary aspects of canine hemophilia is one of the most fascinating now being done on blood coagulation It has helped to dispel the previously held belief that hereditary hemophilia cannot occur in the female as the mating of a hemophilic male and a transmitter female was thought to result in a lethal factor The study of sex linked recessive traits has been hampered in the past because of the scarcity of such traits in mammals The clear demonstration by Dr Brinkhous that the canine hemophilic gene is sex linked and recessive will provide geneticists with a fruitful field for the study of these genes As one who has had many hemophiliacs under his care for the past twenty years we can readily imagine the great pains that have been taken by Dr Brinkhous and his colleagues in raising these animals and maintaining them alive sometimes under trying conditions

I wish to discuss the nature of the defect of coagulation in hemophilic blood and the comparison of the clot promoting power of normal and hemophilic plasma euglobulin *

Human hemophilia falls like many other diseases under three types a) Hereditary that is when more than one generation of patients with the disease are found in a family b) Congenital when the disease dates from birth but no other members of the family are affected c) Acquired a type that has received increasing recognition recently Acquired hemophilia may be seen in the male or female We have under our care at the present time three women with acquired hemophilia all developed the defect after

* This work has been carried on in collaboration with R R Holburn and R T Carroll, Division of Hematology Department of Medicine Jefferson Medical College and Hospital Aided by a grant from the U S Public Health Service

the age of thirty and resemble in most respects the case reported by Fantl and Nance(36) The clotting time of their venous blood is over three hours (silicone tubes) The clotting of the blood and plasma of these patients is accelerated by dilution and behaves in all respects like hemophilic blood The blood and plasma of these patients when subjected concurrently with the blood of hereditary hemophiliacs to most of the tests to measure the various clotting activities respond in all respects like hemophilic blood or plasma and do not do anything that hemophilic blood or plasma fail to do Like most severe hemophiliacs these patients respond in a limited degree to blood transfusions the hemostatic effect of a transfusion of normal blood is slight and transient Curiously the hemophilic women have most of the time little trouble with excessive bleeding during menstrual periods None of the three women had had blood or plasma transfusions previous to developing the hemorrhagic disease

It is important in identifying the hemophilic defect to make sure that no other defects exist in the blood that might account for the delayed coagulation There should be no excessive amount of anti thrombin or fibrinolysin activity The amount of prothrombin Ac globulin and fibrinogen the number and morphology of the platelets should be normal There should be a delay in the venous clotting time when performed in glass tubes sometimes and always when the blood is placed in silicone tubes The prolonged clotting times of venous blood and of plasma should be markedly shortened by dilution with physiological salt solution The conversion of pro thrombin to thrombin should be delayed especially when platelets or cephalin are used as activating agents The response of the plasma to an excess of a thromboplastic extract of brain tissue should be slower than normal particularly when the testing mix ture has a plasma concentration of 70 per cent or higher The slower than normal response of the plasma to platelets thrombo plastin or cephalin should be corrected by dilution of the plasma The bleeding time of shallow cuts of the skin should be normal but that of deep (5 mm or greater) cuts should be prolonged Platelet poor hemophilic plasma should delay the rate of clotting of plate let poor normal plasma in silicone coated tubes and sometimes even in glass tubes Protamine should not shorten significantly the clotting time of the blood or plasma instead it should prolong it

I should like to put several questions to Dr Brinkhous before we go on with our discussion How many of these hemophilic dogs

that received repeated transfusions developed refractoriness to further blood transfusions?

Brinkhous We have not noted a persistent refractory state to transfusions in any of the hemophilic dogs We may have had a temporary refractory state in one animal but it was not studied in sufficient detail at the time to be certain

Tocantins Has Dr Brinkhous observed the appearance at any time of so called antibodies against the clot promoting euglobulins in the serum of these hemophilic dogs?

Brinkhous We have no evidence of this However precipitin and similar tests have not been done on posttransfusion plasma samples

Tocantins Has there been any way of separating the grade of the defect of these animals from the mildest to the most severe on the basis of changes in the blood?

Brinkhous Clinically there are all gradations In the mildest no symptoms were observed until the age of six to ten months In the most severe hemorrhagic symptoms appeared early and frequent transfusions often several per week were needed to keep them alive The clotting time tends to parallel the clinical severity Adding blood of a mild hemophiliac to that of a severe hemophiliac has at the most a slight accelerating effect

Tocantins In the 1949 Transactions of this Conference it was reported by Dr Brinkhous that when platelet poor hemophilic dog plasma was mixed with platelet poor normal dog plasma there was no acceleration of the coagulation Is that not right?

Brinkhous That is essentially correct

Tocantins Would not that particular experiment therefore seem to eliminate the existence of a deficiency in the hemophilic plasma irrespective of any action on the part of the platelets? It is well known that "antihemophilic globulin or "antihemophilic factor" prepared from platelet poor normal plasma promotes the clotting of platelet poor hemophilic plasma this would seem to eliminate the platelets as being necessary to bring about the clot promoting effect of normal plasma on hemophilic plasma

Brinkhous The presence of platelets is needed for the anti hemophilic activity to be manifest Platelets alone are not sufficient Apparently some interaction between the two or possibly two separate actions one by the plasma and the other by the formed elements are needed for the antihemophilic effect of plasma

Tocantins But in the experiments dealing with the rate of prothrombin utilization in hemophilic plasma to which was added small amounts of normal plasma no platelets were present in the system yet prothrombin utilization was accelerated Isn t that right?

Brinkhous Not at all

Tocantins Perhaps I should begin our discussion by stating clearly our point of view regarding the cause of the defect in coagulation in hemophilic blood To our way of thinking hemophilic blood contains all the factors present in normal blood in the normal amount with one exception there is an excess of antithromboplastin This excess is responsible for many secondary changes (increased stability of platelets diminished plasma thromboplastin" or "antihemophilic globulin" delay in conversion of prothrombin incomplete conversion of fibrinogen to fibrin) which have at one time or another been held responsible for the defect in coagulation The following constitutes the evidence to support our point of view

A If platelet poor hemophilic plasma is diluted with 0 85 per cent NaCl it will clot at the same rate as slightly diluted platelet poor normal plasma This is illustrated on Figure 24 in which is

EFFECT OF DILUTION (0 85% NaCl) ON HEMOPHILIC AND NORMAL PLASMAS AND ON NORMAL PLASMA CONTAINING LIPIDANTITHROMBOPLASTIN (L A)

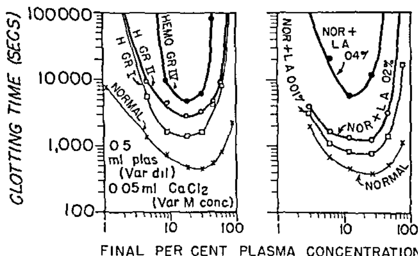


FIGURE 24 Hemo Gr IV H Gr II and H Gr I designate plasmas from hemophilic grade IV hemophilic grade II and hemophilic grade I respectively The lipid antithromboplastin was extracted from human brain (7) and added to the normal plasma to obtain the concentrations shown in the Chart Silicone tubes 38 C The plasma concentrations refer to those in the actual clotting mixtures(27)

shown the effect of dilution on the rate of coagulation of three hemophilic plasmas with defects of different degree of intensity If varying amounts of the lipid antithromboplastin extracted from human blood or brain(37) are added to normal plasma and subsequently each of these slow coagulating plasmas is tested at gradually diminishing plasma concentrations the resulting curves resemble closely those of hemophilic plasmas which have been tested at equivalent plasma concentrations (Figure 24) One can then reproduce the behavior of hemophilic plasma on dilution by taking normal plasma and adding the lipid inhibitor to it By carefully adjusting the amount of purified lipid antithromboplastin it is possible to change normal plasma *ex vivo* so that its behavior towards cephalin thromboplastin platelets thrombin and antihemophilic globulin is indistinguishable from that of natural hemophilic plasma

All the work here mentioned was done on normal or hemophilic human blood or plasma collected processed and tested in silicone coated glassware The anticoagulant generally used was 19 per cent trisodium citrate placed in a siliconized syringe (0 2 ml of the citrate solution 10 ml of blood) into which the blood was aspirated In the calculation of the final per cent concentration of the plasma in the actual clotting mixtures any or all of the following variables were taken into account a) the per cent plasma volume of the sample of blood (calculated from the hematocrit) b) The total volume of anticoagulant added to the blood c) The total volume of the clotting mixture itself and the respective volumes of each of its ingredients namely plasma recalcifying solution activating agent and diluting fluid(27)

B Dilution gradually reduces and finally eliminates the difference between normal and hemophilic plasma whether activated by platelets or aqueous extracts of brain tissue or by cephalin

If washed normal or hemophilic platelets suspended in 0 85 per cent NaCl are added to hemophilic plasma it is possible to accelerate the rate of coagulation of this plasma until it reaches the normal range(38) Between eight and ten times more platelets than are present normally in plasma are necessary to bring this about When the number of platelets in the clotting mixture is however about 1 200 000 per c mm (see Figure 25) the hemophilic plasma at 74 per cent concentration takes almost twice as long to clot as 74 per cent normal plasma If the concentration

EFFECT OF THE ADDITION OF WASHED HUMAN PLATELETS ON THE RATE OF COAGULATION OF DILUTED (0 85% NaCl) NORMAL & HEMOPHILIC PLASMA

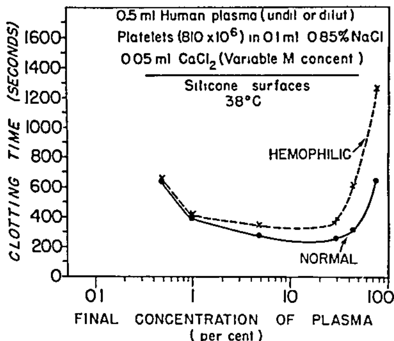


FIGURE 25

of the plasma is gradually reduced while maintaining number of platelets and volume of the mixture constant the rate of coagulation of the two plasmas is accelerated and the difference between the two rates is gradually reduced until when a clotting mixture with a plasma concentration of about 1 per cent is reached the two plasmas clot at the same rate (Figure 25) Addition of distilled water extracts or destroyed platelet suspensions (instead of intact platelets) to diluted hemophilic or normal plasma does not alter the results significantly

Essentially similar results are observed when a potent thromboplastin prepared from an aqueous extract of human brain is added to hemophilic and normal plasma In the clotting mixtures of 74 per cent plasma concentration hemophilic plasma clots slower than normal plasma when activated by this strong thromboplastin If while maintaining thromboplastin concentration and the *total volume of the mixture constant* the plasma concentration is reduced a point is reached (at about 10 per cent plasma concentration) when the two plasmas clot at the same rate(27)

It has been known for sometime that cephalin is a poor activator of coagulation of hemophilic plasma (39) As shown in Figure 26 when hemophilic plasma is diluted to a concentration of 5 per cent it clots after addition of cephalin as fast as normal plasma

Brinkhous How was the cephalin prepared?

Tocantins The cephalin was prepared by precipitating it with cold absolute ethanol from the ether extract of acetone dried human brain The cephalin was purified by repeated precipitations with absolute ethanol (40)

C Sufficient exposure of hemophilic plasma to contacting sur faces such as asbestos fibers or kaolin will change it so that its rate of coagulation and behavior on dilution will equal that of normal plasma Stable normal and hemophilic citrated plasmas were exposed to loose asbestos fibers (10 mg asbestos per 1 ml plasma in silicone coated tubes without agitation at 20 C) for varying periods of time (zero to two hundred ten minutes) After

EFFECT OF DILUTION (0 85% NaCl) OF PLASMA (NORMAL AND HEMOPHILIC) ON THE CLOT ACCELERATING ACTION OF CEPHALIN

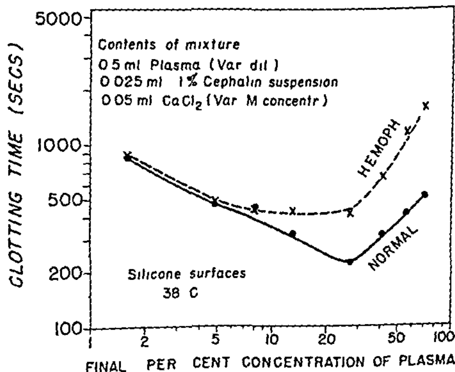


FIGURE 26 Cephalin prepared from acetone dried human brain (40)

the stipulated interval the fibers were picked down at the bottom of the tube the plasma removed and its response to dilution tested at once While stable normal and hemophilic plasmas (not exposed to asbestos) when tested in a mixture of high plasma concentration (89 per cent) took longer to clot than when diluted tenfold after the plasmas had been in contact with asbestos for one hundred and twenty minutes their clotting times were much shortened and dilution did not have as great an effect in accelerating their clotting rates The concentrations of prothrombin and Ac globulin in the exposed plasmas as measured by the two stage method(41 42 43) were not significantly altered Hemophilic plasma which when tested in a mixture above 50 per cent plasma concentration did not clot before exposure to asbestos clotted after sixty minutes of contact Two hundred and ten minutes of contact were required for hemophilic plasma to reach the rate of coagulability of normal plasma exposed for only one hundred and twenty minutes (Figure 27) As after addition of platelets cephalin and thromboplastin the rate of coagulation of normal and hemophilic plasma may also be equalized by appropriate exposure to a suitable contacting surface

Waugh Does asbestos remove fibrinogen?

EFFECT OF CONTACT WITH ASBESTOS FIBERS ON THE RESPONSE OF NORMAL AND HEMOPHILIC PLASMA TO DILUTION

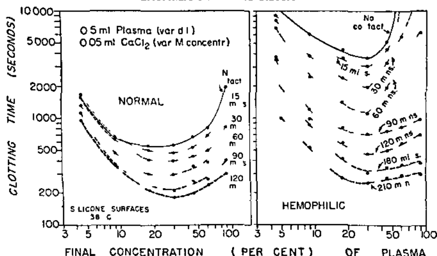


FIGURE 27 Each specimen of plasma was kept in contact with asbestos fibers for the allotted period of time before the asbestos was removed and the response of the plasma to dilut on tested

Tocantins Yes There is a decrease of about 30 per cent of the fibrinogen when 100 mg of asbestos per milliliter of plasma are used for one hour When 10 mg or less of asbestos per ml plasma are employed and there is no shaking no significant decrease in the fibrinogen results

D Dilution accelerates the rate of prothrombin conversion in normal and hemophilic blood If normal blood is placed in silicone coated tubes and the rate of conversion of its prothrombin before and during clotting is closely followed a curve like that shown in Figure 28 is obtained if the blood is diluted with an equal amount of 0 85 per cent NaCl conversion of prothrombin begins earlier When hemophilic blood is diluted 1 to 1 with 0 85 per cent NaCl immediately after its collection prothrombin conversion may start one hour or longer before it does in undiluted hemophilic blood (Figure 28) (Table VIII)

RATE OF PROTHROMBIN CONVERSION IN COAGULATING NORMAL AND HEMOPHILIC BLOOD UNDILUTED (100%) AND DILUTED (1 1) WITH 0 85% NaCl (50%)

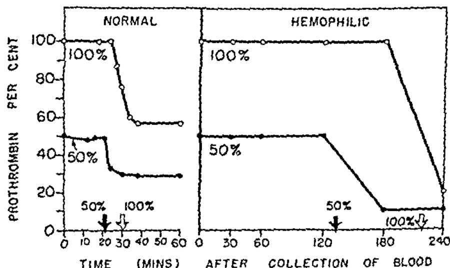


FIGURE 28 Prothrombin measured by the two-stage method(41) Blood allowed to stand in silicone coated tubes at 38° C during clotting The rate of prothrombin utilization was measured as described by Brinkhous(43) The white arrows at bottom of chart indicate the time at which the undiluted blood clotted black arrows the time when the 50% blood clotted

These findings are incompatible with the explanations that the delayed coagulation of hemophilic blood results from an insufficiency of an accelerator ("antihemophilic globulin")(44) or the precursor of an accelerator ("thromboplastinogen")(25) or a plasma

TABLE VIII

Time of Start of Prothrombin Conversion of Undiluted (100 per cent) and Diluted (50 per cent) Normal and Hemophilic Blood

	NORMAL					HEMOPHILIC		
Specimen No	1	2	3	4	5	1	2	3
Time (Mins) Required for Start of Prothr Conversion								
Blood (100 per cent)	25	20	23	18	18	180	240	120
Blood (50 per cent)*	15	15	21	10	15	120	180	60

* Blood diluted 1 1 with 0 85 per cent NaCl immediately after collection Silicone Tubes 38 C

TABLE IX

Effect of Diluting Two Samples of Hemophilic Plasma With Either Normal Plasma or 0 85% NaCl, on the Clotting Time of the Mixture Silicone Surfaces, at 38 C

Hemophilic Plasma (ml)	Normal Plasma (ml)	0 85% NaCl (ml)	$CaCl_2$ (ml)	CLOTTING TIME (Seconds)	
				Hemophilic #1 (Grade IV)	Hemophilic #2 (Grade II)
0 25	—	0 275	0 025 (0 2 M)	82 000	4 735
0 17	—	0 21	0 17 (0 02 M)	6 230	3 360
0 1	—	0 35	0 1 (0 02 M)	4 770	2 885
0 5	—	—	0 05 (0 2 M)	>100 000	>100 000
0 25	0 25	—	0 05 (0 2 M)	>100 000	4 295
0 17	0 33	—	0 05 (0 2 M)	>100 000	4 100
0 1	0 4	—	0 05 (0 2 M)	>100 000	3 890

Clotting time of 0 5 ml Normal Plasma plus 0 05 ml 0 2 M CaCl = 1170 seconds

factor necessary for the mobilization of the clot accelerating factor in platelets(23) If any or all of these deficiencies existed in hemophilia dilution of the blood or plasma would naturally accentuate them further delay the rate of coagulation and widen the difference between the two bloods However such is not the case Indeed the clotting time of hemophilic plasma moderately diluted with 0 85 per cent NaCl is usually shorter than when "diluted" with comparable volumes of stable normal plasma (Table IX)

E Hemophilic plasma has a clear clot delaying action on normal plasma If high plasma concentrations are maintained in the actual clotting mixtures and the plasma is collected and kept in silicone coated tubes this effect can be readily demonstrated In Table X are shown the results of the addition of hemophilic plasma to normal plasma As little as one part of hemophilic plasma when added to nine parts of normal plasma may suffice to prolong the rate of clotting of normal plasma significantly A significant clot delaying effect is not considered to exist unless one part or less of hemophilic plasma prolongs the clotting of three parts or more of normal plasma by at least one third the clotting time of an equal volume of whole normal plasma As shown in Table X this is true of all four hemophiliacs listed The intensity of the hemophilic defect and the surface of the vessel in which the tests are conducted determine the minimum amount necessary to delay the clotting time of normal plasma Hemophilic plasmas that exert a clear clot delaying action in silicone or collodion tubes may fail to do so when the tests are carried out in glass tubes Likewise if an artificial anticoagulant plasma (normal plasma to which antithromboplastin has been added) is tested in glass tubes for a clot delaying effect on normal plasma only those artificial anticoagulant plasmas containing an excess of anticoagulant will exert such an effect but if the tests are conducted in collodion or silicone tubes even the plasmas with a low anticoagulant content will exert a clot decelerating effect (Figure 29)

It is likely that some of the presumably abnormal circulating anticoagulants" that have been recently reported to appear in the blood of previously normal persons and of hemophiliacs represent in many instances simple increases of the natural plasma antithromboplastin to a level where the presence of an anticoagulant may be detected even when glass tubes are employed It is significant that more than one half of the patients reported to have "circulating anticoagulants" have been hemophiliacs(45 46) who

TABLE X

Effect of Addition of Hemophilic Plasmas of Varying Degrees of Severity, on the Rate of Clotting of Normal Plasma Silicone Tubes 38° C

Tube No	Normal Plasma (ml)	Hemoph Plasma (ml)	0.2 M $CaCl_2$ (ml)	*HEMOPHILIC PLASMA* Grade I (Mild)	Grade II (Moderate)	Grade III (Severe)	Grade IV (Very Severe)
				clotting time in seconds			
1	0.5	0.0	0.05	1850*	2050*	1450*	1470*
2	0.45	0.05	0.05	2850	3250	3700	82 000
3	0.4	0.1	0.05	3250	3700	86 000	No clot
4	0.3	0.2	0.05	4400	5400	No clot	No clot
5	0.2	0.3	0.05	6500	9300	No clot	No clot
6	0.1	0.4	0.05	41 000	No clot	No clot	No clot
7	0.0	0.5	0.05	No clot	No clot	No clot	No clot

* Each normal plasma came from a different donor

Plasma A Normal plasma Plasma B Normal plasma + 0.7% lipid antithromboplastin. Plasma C Normal plasma + 0.24% lipid antithromboplastin

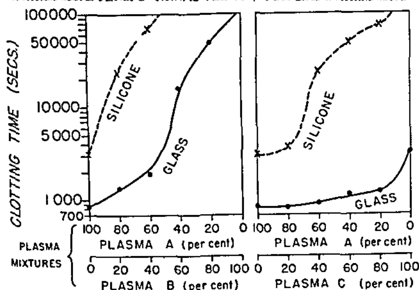


Figure 29 Clot decelerating action of two artificial anticoagulant plasmas (plasma B and C) when mixed in various proportions with normal plasma and tested in glass or silicone tubes Anticoagulant plasma prepared by adding a small volume of a strong lipid antithromboplastin to normal plasma Each clotting mixture had a final plasma concentration of 72 to 75%

already have an abnormally high amount of a similarly acting naturally occurring anticoagulant It seems more proper to speak of "excessive circulating anticoagulants to describe such cases inasmuch as all of us have anticoagulants (antithromboplastin antithrombin) normally circulating in the blood We have found an increase in plasma antithromboplastin (anticephalin) activity as a complication of diseases such as sickle cell anemia leukemia disseminated lupus erythematosus and following dicumarol therapy The hypocoagulability and occasional clot delaying effect of the blood of animals exposed to ionizing radiations are probably due in part to the entrance of antithromboplastin in excessive amounts into the circulating blood

F If a normal euglobulin solution prepared from 10 per cent plasma is tested on both normal and hemophilic plasmas in a clot ting mixture of high plasma concentration it has a definite clot accelerating effect on both plasmas although the response of the hemophilic plasma is somewhat slower As the concentration of the euglobulin solution is reduced the clot acceleration is less

RESPONSE OF NORMAL AND HEMOPHILIC PLASMAS TO NORMAL AND HEMOPHILIC GLOBULIN FRACTIONS

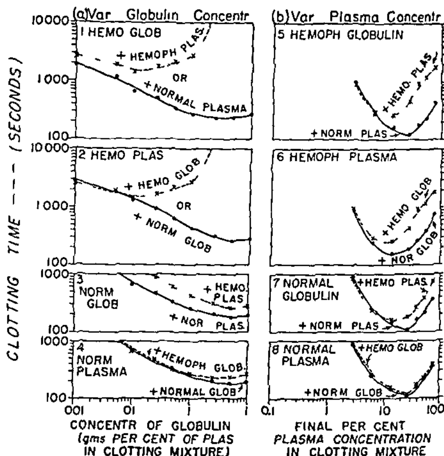


FIGURE 30 Contents of clotting mixtures a) Variable globulin concentration 0 5 ml plasma 0 1 ml euglobulin (variable dilution) 0 05 ml 0 2 M $CaCl_2$ b) Variable plasma concentration 0 5 ml plasma (variable dilution with 0 85% NaCl) 0 1 ml euglobulin (0 4%) 0 05 ml CaCl (Variable M concentration) Silicone 38 C

marked *Hemophilic euglobulin loses its clot accelerating effect to the same degree as the normal euglobulin when tested on normal* plasma On hemophilic plasma however the hemophilic euglobulin (prepared from 10 per cent hemophilic plasma) has no apparent clot accelerating effect when used in high concentration (Figure 30 No 1) when the euglobulin solution is diluted it *gains* clot accelerating activity until a point is reached (at about 0 01 gram per cent of the euglobulin) at which *both normal and hemophilic euglobulins accelerate the clotting of hemophilic plasma to the same degree* (Figure 30 No 2) The euglobulin solutions are pre

pared by diluting the plasma with cold distilled H_2O, adding 1 per cent acetic acid to pH 6.0, centrifuging the precipitate and washing it with distilled H_2O. The precipitate is dried, weighed and dissolved in 0.85 per cent NaCl and the pH adjusted to 7.4.

If a normal euglobulin solution of constant concentration is added to plasma of varying concentration, clotting is accelerated as plasma concentration is reduced. Though the response of almost undiluted hemophilic plasma to the normal euglobulin is slower than that of almost undiluted normal plasma, the two plasmas respond alike to the euglobulin when a plasma concentration of about 12 per cent is reached (Figure 30, No. 7). The same happens when the amount of hemophilic euglobulin is kept constant while the two plasmas are gradually diluted, though lower plasma concentrations must be reached (5 per cent) before the hemophilic euglobulin will bring about clotting of both normal and hemophilic plasmas at the same rate (Figure 30, No. 5).

C. The clot accelerating activity of hemophilic and normal euglobulin prepared from adequately diluted plasmas is alike. Hemophilic and normal plasmas were diluted with cold distilled water to obtain varying concentrations of the plasmas ranging from 20 to 0.1 per cent. Each dilution was then adjusted to a pH of 6.0 with 1 per cent acetic acid and allowed to stand for about one hour; the precipitates were then centrifuged, dried, weighed and dissolved just before testing in 0.85 per cent NaCl, the pH being adjusted to 7.4.

The euglobulins separated by acidification of more concentrated plasma samples (10 to 20 per cent concentration) have less clot accelerating effect on both normal and hemophilic plasma than those made from more dilute plasma (2 per cent or lower concentration). Although the euglobulin fractions prepared from high concentrations (10 per cent) of hemophilic plasma are markedly less active as accelerators of hemophilic plasma than the fraction made from a similar concentration of normal plasma, the two euglobulins gradually approach each other in potency as more dilute plasmas are employed in preparing them. When prepared from 0.2 per cent plasmas the clot promoting activities of the normal and hemophilic euglobulin on hemophilic plasma are alike (Table XI).

Another experiment supports the explanation that the euglobulin fraction can be dissociated from inhibitors by adequate dilution of the plasma before the euglobulin is precipitated. If enough lipid antithromboplastin is added to normal citrated plasma (designated

TABLE XI

Acceleration of the Clotting of Hemophilic Plasma By Addition of Euglobulin Solutions Prepared From Various Concentrations of Normal and Hemophilic Plasma

Clotting Mixture	Concentration of Plasma (%) Used For Preparation of Euglobulin				
	20	10	2	0 2	0 1
	clotting time in seconds				
1 0 5 ml Hemo Plasma 0 1 ml Normal Euglobulin (0 4%) 0 05 ml 0 2 M CaCl	475	420	358	355	374
2 0 5 ml Hemo Plasma 0 1 ml Hemo Euglobulin (0 4%) 0 05 ml 0 2 M CaCl	5400	2860	945	365	360

0 5 ml hemophilic plasma plus 0 05 ml 0 2 M $CaCl_2$ = No clot in 24 hours

TABLE XII

Acceleration of Clotting of Hemophilic Plasma by Euglobulin Solutions Prepared From Varying Concentrations of Normal and an Artificial Anticoagulant Plasma

Clotting Mixture	Concentration of Plasma (%) Used For Preparation of Euglobulin			
	10	2	0 2	0 1
	clotting time in seconds			
1 0 5 ml Hemo Plasma 0 1 ml Normal Euglobulin (0 4%) 0 05 ml 0 2 M CaCl	495	252	155	140
2 0 5 ml Hemo Plasma 0 1 ml Euglob (0 4%) from anticoag Plasma 0 05 ml 0 2 M CaCl	1460	348	145	140

0 5 ml hemophilic plasma plus 0 05 ml 0 2 M CaCl = No clot in 24 hours

in Table XII as anticoagulant plasma) its clotting time may be prolonged over tenfold The activity of euglobulins precipitated from such plasmas is much less than that from the parent normal plasma if the plasmas are diluted only 1 to 10 before the euglobulin is precipitated As greater dilutions are used the resulting euglobulins whether prepared from normal or anticoagulant plasmas eventually display the same activity when tested on hemophilic plasma as a substrate (Table XII)

In vivo work now in progress corroborates these results namely that euglobulin solutions prepared from suitably diluted hemophilic plasma are as effective when injected intravenously as those prepared from a 1 to 10 diluted normal plasma in accelerating the clotting of hemophilic blood The well known fleeting nature of the clot accelerating effect of these euglobulin solutions as well as of transfusions of normal blood is probably due to an inactivation of the injected euglobulins by the recipient's own plasma This is supported by the fact that the clot accelerating effect of a euglobulin solution separated from normal plasma is reduced by incubation *in vivo* with normal plasma and almost nullified by appropriate incubation with hemophilic plasma On Figure 31 is shown the extent of these changes Adequate dilution of normal plasma or heating it (65 C ten minutes) will destroy its euglobulin inactivating effect In order to destroy completely this property in hemophilic plasma higher temperatures for longer periods are necessary than those required for normal plasma

II Extraction of hemophilic blood and plasma has yielded a mean of units of antithromboplastin per ml of blood over seven times higher in the hemophilic group than in the normal(47) The assay for antithromboplastin activity of hemophilic and normal tissues revealed a higher content in hemophilic tissue though with the exception of skeletal muscle the thromboplastic activity of saline extracts of hemophilic and normal tissues were about the same(18)

These and other points elaborated in greater detail elsewhere(38) are obviously in conflict with the current theories purporting to explain the retarded coagulation of hemophilic blood as resulting from a deficiency or absence of a coagulant or procoagulant factor

Flynn Can you treat a hemophiliac with his own blood?

Tocantins It can be done in one of two ways One pint of citrated blood is collected in glass bottles from a hemophiliac and

TABLE XI

Acceleration of the Clotting of Hemophilic Plasma By Addition of Euglobulin Solutions Prepared From Various Concentrations of Normal and Hemophilic Plasma

Clotting Mixture	Concentration of Plasma (%) Used For Preparation of Euglobulin				
	20	10	2	0.2	0.1
	clotting time in seconds				
1. 0.5 ml Hemo Plasma 0.1 ml Normal Euglobulin (0.4%) 0.05 ml 0.2 M CaCl	475	420	358	355	374
2. 0.5 ml Hemo Plasma 0.1 ml Hemo Euglobulin (0.4%) 0.05 ml 0.2 M CaCl	5400	2860	945	365	360

0.5 ml hemophilic plasma plus 0.05 ml 0.2 M $CaCl_2$ = No clot in 24 hours

TABLE XII

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Clotting Mixture	Concentration of Plasma (%) Used For Preparation of Euglobulin			
	10	2	0.2	0.1
	clotting time in seconds			
1. 0.5 ml Hemo Plasma 0.1 ml Normal Euglobulin (0.4%) 0.05 ml 0.2 M CaCl	495	252	155	140
2. 0.5 ml Hemo Plasma 0.1 ml Euglob (0.4%) from anticoag Plasma 0.05 ml 0.2 M CaCl	1460	348	145	140

0.5 ml hemophilic plasma plus 0.05 ml 0.2 M $CaCl_2$ = No clot in 24 hours

Another method is to infuse hemophilic plasma that has been in contact with sterile asbestos fibers for a fixed period of time without agitation Such a plasma will accelerate the clotting of hemophilic blood *ex vivo* and *in vivo*

Brinkhous There has been the suggestion of using plasma from aged whole hemophilic blood for transfusing hemophiliacs Have you tried that?

Tocantins No I believe that has been tried by others

Brinkhous There are conflicting reports on its values

Such aged preparations are without effect in our animals

Tocantins I don t think one can get as hypercoagulable a plasma by just allowing hemophilic plasma to stand as by placing it in contact with a surface like asbestos fibers or for that matter barium sulphate and other so called adsorbents which supposedly do only one thing — remove prothrombin from plasma

I S Wright Dr Haberman —

Haberman The genetic studies presented by Dr Brinkhous on these hemophilic dogs are the most complete that I have ever seen To begin this short discussion of the problem of hemophilia I should point out that our work at the Baylor University Graduate Research Institute was an outgrowth of some studies on erythro blastosis and the determination of heterozygosity in the inheritance of the Rh antigens The successful outcome of such experiments led our staff to consider the possibility that laboratory methods might reveal something about heterozygosity in the carrier female of hemophilia The work was done by Joseph M Hill John Ellis Gwendolyn Crass and Kenneth Wittstruck(19) My part in this report was that of amateur geneticist and immuno hematologist for blood group studies on all families in the series Many of the blood clotting tests available at the time were applied to the examination of blood samples from families in which hemophilia occurred The tests included bleeding time clotting time prothrombin levels prothrombin utilization platelet counts Factor V (Ac globulin) and the slow and fast recalcification techniques In addition to these tests the erythrocytes of each member of the family was studied serologically to determine the blood antigens These studies afforded a means of lineage control and in two instances nonpaternity was uncovered The sixteen antisera used for this study were Anti A B absorbed B M N C D, E, c, d, e, C^w P Lu Le and Kell

CLOT PROMOTING ACTIVITY OF NORMAL EUGLOBULIN AFTER INCUBATION WITH 4 TYPES OF PLASMA

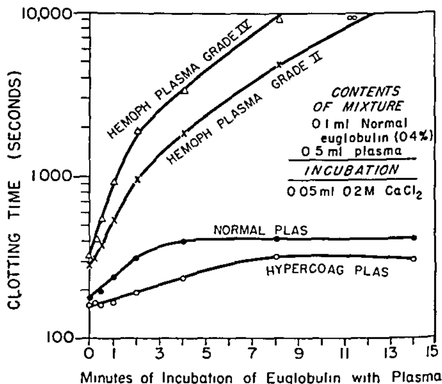


FIGURE 31 The hypercoagulable plasma was from a patient after a gastrointestinal hemorrhage Prothrombin and Ac globulin of all plasmas within normal limits Tests done in silicone tubes 38 C

the plasma is separated by centrifugation Using a technique to preserve sterility throughout 200 to 300 ml of the plasma is diluted 1 to 100 or 1 to 200 or higher with cold sterile water and the pH adjusted to 6 0 with 1 per cent acetic acid The resulting precipitate is centrifuged down and the residue dissolved just before injection in 200 to 300 ml of 0 85 per cent NaCl pH adjusted to 7 4 The solution is filtered while cold through a Berkefeld V filter and infused intravenously over a thirty minute interval We have administered euglobulin solutions so prepared to hemophiliacs before a tooth extraction The changes in clotting time and the hemostatic effect produced are in most respects comparable to those obtained from infusions of euglobulins prepared from 1 to 10 diluted normal plasma

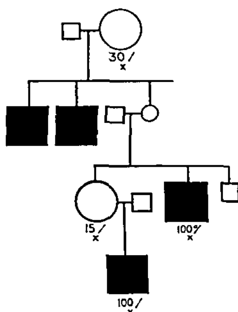


FIGURE 32. Family McW Jo showing prothrombin utilization defect in the carrier females (Data given on prothrombin utilization Carrier and hemophilic progeny drawn larger than untested or normals In this figure as in the others % refers to the residual prothrombin of the serum Figure 35 shows the meaning of the other symbols

showed a defect that was detected by the Quick prothrombin utilization test It is quite possible that this may be a test for heterozygosity and in this family it seemed to fit very nicely with the accepted theory of recessive sex linkage of hemophilia

Quick May I ask about that? You have a carrier a normal male and then you have two hemophiliacs?

Haberman Yes I should emphasize at this point that not all of the individuals shown were tested Only those marked with prothrombin utilization values were studied in this family There was a carrier female an untested male and a progeny containing two hemophiliacs We were unable to obtain blood from the second generation female and the male she married but as far as we know he had no history of hemophilia in his family This family is typical of hemophilia and is quite representative of what we found at first However when other families were studied more completely the prothrombin utilization data became significant

During the early part of this study the one stage prothrombin utilization (25) test gave some interesting results especially in the cases of possible female carriers of hemophilia At this time I would like to emphasize that I do not wish to equivocate or enter into argument on the specificity of the one stage method We do not claim that this method determines prothrombin as such but we did find some *interesting results that fit in with the discussions in* progress

In considering the well known and accepted genetics of hemophilia one is soon faced with some of the statements that have been made by Haldane(50) He has calculated that on the average a hemophilia gene would die out in approximately three generations in a stable population However Gates(51) reviews a number of instances in which hemophilia persists for more than three generations If one third of the hemophilic genes are lost in each generation one can conceive of the eventual disappearance of hemophilia Haldane considered this possibility in the light of data that showed the incidence of hemophilia in London to be constant He therefore surmised that the rate of mutation to hemophilia must be equivalent to the frequency of hemophilia in order to replace the lost hemophilic genes He estimated the rate as approximately 1 in 50 000 in the London population The evidence for such mutations is found in families with no obtainable history of hemophilia producing progeny having the disease This would mean that there is a tendency for mutation of a normal gene resulting in the disease hemophilia There are a number of instances in genetics in which a point mutation might explain such a situation With these theories in mind we began our studies on hemophilia with the hope of detecting the heterozygous female carrier Some of the results can be seen in the following charts In Figure 32 is seen a part of the family McW Jo in which hemophilia appeared without any previous history The first progeny in the four generations shown resulted in two cases of hemophilia The third generation had one case and the fourth generation also had one case

When the various tests were applied to the available members of the family *it was found that the mother of the first generation* had a prothrombin utilization value of 30 per cent 20 per cent greater than our normal range In generation three the female had a prothrombin utilization value of 15 per cent and the hemophilic male showed 100 per cent unutilized prothrombin as did the male of the fourth generation It was felt that the two females tested

In Figure 33 is family Max Foy The data shown covers four generations with thirty six individuals having been studied Of these two were clinical hemophiliacs and fourteen showed ab normally high unutilized prothrombin by the Quick prothrombin utilization test One male had died of hemophilia before this study began In this family one can readily observe the prothrombin utilization abnormality on both sides of the family Both the grand mother and mother of the hemophiliacs had the defect The two living hemophilic males showed 100 per cent unutilized prothrombin and the only daughter had the defect It is interesting to note that the father in this case had a normal prothrombin utilization of 10 per cent However his sisters had the defect to the extent of 22 per cent and 27 per cent unutilized prothrombin It can be seen that in both sides of the family the defect can be found in the males as well as females

In Figure 34 is family Cam Tam showing five generations of whom thirty six members were tested The defect is seen in both males and females on both sides of the family In addition both parents of the hemophilic male demonstrated the defect The heavy double line indicates that the parents of the hemophiliac were distant cousins This family is quite typical of the results we have been finding Not only do both sides of the family have this defect but frequently both parents have it

Alexander What is the defect in the male on the left the father of the nonhemophiliac?

Haberman Twenty per cent unutilized prothrombin

Tocantins What are the mean values for normal blood?

Haberman In our study on normals we ran a series of blood bank donors and found that the normals ran between five and ten per cent by our tests We arbitrarily added five per cent and used zero to fifteen as our normal range

Alexander That is recalcified bank blood?

Haberman No The blood was taken directly from the donor after the bank collection After the blood bank bottle was discon nected the blood for this study was obtained

Alexander And what interval after the blood was shed was the serum withdrawn and subjected to determination?

Haberman I think that was immediately We took it immediately to the laboratory and started work on it

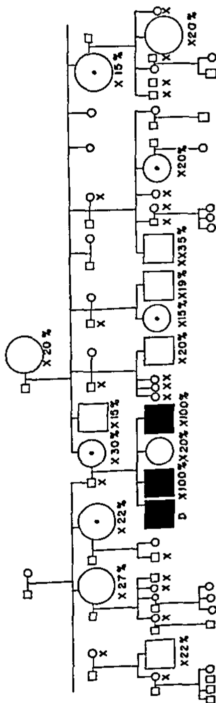

PEDIGREE OF HEMOPHILIA — FAMILY MAY FOY

FIGURE 33

In Figure 33 is family Max Foy The data shown covers four generations with thirty six individuals having been studied Of these two were clinical hemophiliacs and fourteen showed ab normally high unutilized prothrombin by the Quick prothrombin utilization test One male had died of hemophilia before this study began In this family one can readily observe the prothrombin utilization abnormality on both sides of the family Both the grand mother and mother of the hemophiliacs had the defect The two living hemophilic males showed 100 per cent unutilized prothrombin and the only daughter had the defect It is interesting to note that the father in this case had a normal prothrombin utilization of 10 per cent However his sisters had the defect to the extent of 22 per cent and 27 per cent unutilized prothrombin It can be seen that in both sides of the family the defect can be found in the males as well as females

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PEDIGREE OF HEMOPHILIA – FAMILY CAM TAM

FIGURE 34

Alexander I am a little confused Just the serum was obtained from the blood How long was it permitted to clot before it was tested?

Haberman I am not sure Dr Alexander I know they collected that blood from the donor and then immediately took it to the laboratory to study

Alexander And the carrier male that showed the defect had consumed only 20 per cent of his plasma prothrombin?

Haberman Yes

Alexander Is there is a history of hemophilia in the family of the father of the hemophiliac?

Haberman No there is no history of hemophilia In this family we have data on four generations before the hemophiliac occurred

Alexander The point I wish to bring up is that there are a number of people who consider that the lack of prothrombin consumption or utilization is a test for thromboplastinogen too

Haberman We are not making claims as to what this test actually detects We are reporting this data occurs in these families histories

Alexander A presumptive test more or less?

Haberman Yes we think it might be

Warner After the blood was shed and clotted and before the residual prothrombin was tested at the laboratory was the time after clotting standard or might the post clotting time have varied from a few minutes to an hour or two before the test was actually made?

Haberman The test used was the Quick one stage method The observations were made at one three and twenty four hour intervals The twenty four hour data on serum is all that is given in these figures

Warner It was a standard time?

I S Wright Looking at this in a different way suppose you took ten families and tested as many individuals in ten families none of whom had hemophilia what would be your incidence of tests similar to these that you consider to be suggestive tests?

Haberman Zero

Alexander As I recall two or three years ago we published on the residual serum prothrombin obtained from one hundred presumably normal individuals and I think that about 5 or 10 per cent of the so called normal individuals had serum prothrombin levels by the one stage method as high as 20 per cent

Haberman In our normal controls it did not go over 10 per cent We ran one hundred consecutive blood bank donors and found none over 10 per cent During the study it was thought that conditions such as pregnancy might influence the results A series of women in the maternity division was tested but they also fell within the normal range

Alexander Well then I think another of the important variables that must be considered is the time interval after the blood is shed to the time that the serum is measured for its prothrombin activity

Haberman The team that made the family studies aimed for standard times of one three and twenty four hours for prothrombin utilization testing To do this the team was provided with a station wagon equipped as a mobile laboratory The families to be studied would congregate at one of the homes and much of the work was done at this site

Quick It is important also to emphasize that the tests should be repeated a number of times on the same individuals because according to our studies the factor which we call thromboplastinogen does not stay constant in normal individuals It varies It s unlike the prothrombin which remains constant

Jaques I might reply to Dr Alexander s question on the incidence by giving my calculations based on Dr Haberman s table The incidence is about 20 per cent of individuals showing defects which is considerably above the incidence you discovered in the general population

Haberman The data presented in Figure 35 shows a negro family in which hemophilia occurred This chart shows the family tree based on information obtained from members of the family The prothrombin utilization results and the detailed blood group data are given

There was no previous history of hemophilia in this family The sudden appearance of a hemophiliac fits with the literature on the rareness of this disease in the negro One might assume that in this

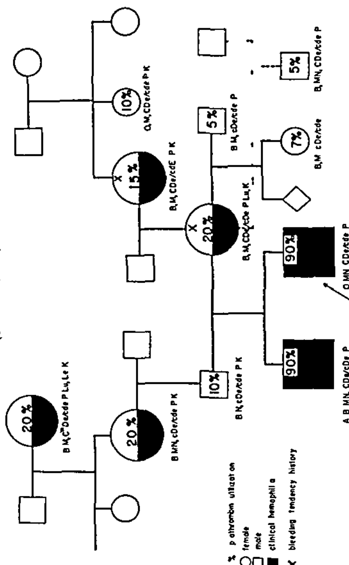


FIGURE 35

family a mutation brought on this situation However when the prothrombin utilization data is examined one may readily see the defect on both sides of the family It is also interesting to note that the supposed father of the hemophiliacs had a normal prothrombin utilization while the defect was present in his mother and grand

mother The marriage with a female having the defect resulted in hemophilia

The normal prothrombin utilization of this father along with other such instances has made us consider the possibility of another substance having normalizing or neutralizing effect in the case of carriers The presence of this gene (neutralizing) might suppress the appearance of the prothrombin utilization defect

The history of this family is somewhat amusing The mother was free with her information and readily answered any questions *concerning her family and children Her present marriage resulted* in two children both of whom were hemophiliacs Before this marriage she had another husband That marriage resulted in two children one normal and living and the other died of causes unknown but apparently not hemophilia In addition the mother admitted extramarital relations with a third man by whom she had a normal son

Tocantins Was that confirmed serologically?

Haberman We studied this family serologically and Figure 36 answers your question The prospectus (the child brought in for treatment which led to the family study) could be the offspring of the second marriage but the other hemophilic child could not be the issue of the present husband or the previous one Consequently we must assume that he is either the son of the admitted paramour or possibly a fourth man This case emphasizes one of the dangers that is faced when one is doing genetic studies on any disease even though the mother is trying to give you the whole story In this case she did not know the whole story or didn t remember

The prothrombin utilization test in our hands seems to be an indicator of the carrier state of a clotting defect associated with the disease hemophilia I would not care to enter into a discussion on the mechanism involved The finding of this defect in both males and females raises certain questions on the inheritance of hemophilia in man If these findings are valid and I suppose this is a hypothetical question then it would be difficult to call hemophilia a sex linked disease There are two other types of inheritance that should be considered one is a sex influenced character and the other is a sex limited character

In the sex influenced characters *baldness in man* is a good example In this case the condition is due to the inheritance of a

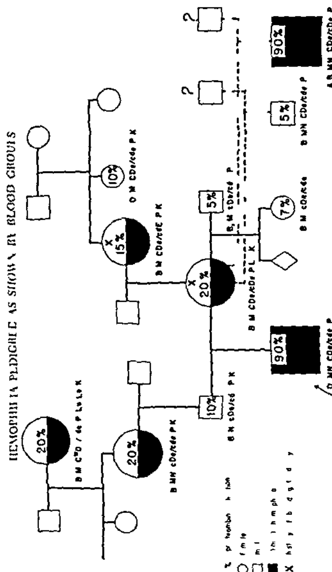


Figure 38

dominant gene "B." In man the presence of one "B" on the chromosome locus results in baldness. However, in the female a single heterozygous "B" results in the carrier condition but not baldness. The *female must be homozygous to develop the bald condition*.

TABLE XIII

Genes	Female	Male
b b	normal hair	normal hair
B b	normal hair	bald
B B	bald	bald

Apparently, sex has an influence on the heterozygous condition Bb suppressing the bald character in the case of the female When the female inherits two bald genes BB the bald condition over comes the influence of sex

In the second type of inheritance that might be considered sex limited characters the feathers of chickens is a good example In this case the cock type feathers are inherited at one locus by a recessive gene 'b The hen feathers are due to the dominant H" gene as follows

TABLE XIV

Genes	Female	Male
H H	hen feathers	hen feathers
H h	*hen feathers*	*hen feathers*
h h	hen feathers	cock feathers

No matter what genes are inherited by the female hen type feathers result But in the case of the male cock feathers are pro duced when the recessive gene h" occupies both sides of the chromosome locus The proof that sex is the limiting factor was demonstrated by the surgical removal of the ovaries of young chicks This resulted in hens with cock feathers Now if the genetics of hemophilia is not sex linked then one must consider sex influenced and sex limited inheritance Also in order to do adequate genetic studies in humans careful serologic paternity studies must be done

Laki I would like to ask Dr Brinkhous if he has any idea about the chemical nature of the hemophilic factor Does it seem to be a protein?

Brinkhous In my opinion it is a plasma euglobulin

Laki In this connection I would like to mention experiments my pupil Lórand and I did some years ago We found that kaolin

absorbs this factor from normal plasma acidified to about pH 6 Strong borax solution eluated this factor from the kaolin The eluate contained very little protein which shows that it must be a very active agent if it is protein We used the simple clotting time test with hemophilic blood or plasma to test the activity of this factor and found that the heat destruction of the hemophilic factor has a high temperature quotient just like the heat destruction of any protein Unfortunately we had to give up work at that stage

Ferguson I should like to report some experiments (Table XV) we made last week on the effects of certain proteolytic enzymes on the clotting of recalcified citrated plasma of the hemophilic dog obtained through the cooperation of Dr Brinkhous A comparison is made between A) enzymes — (tests 4) crystalline trypsin (0 0025%) (tests 5) commercial trypsin (0 1%) (tests 6 7 8) three different preparations (I II III) of dog serum fibrinolysin (tests 9) Loomis bovine serum fibrinolysin B) protease free "thromboplastic" agents — (tests 2) brain thromboplastin (Schieffelin s) (tests 3) purified brain cephalin (0 1%) (tests 10) dog platelet extract C) saline controls — (tests 1 1a) Clotting times at 37°C were determined in the presence of (a) saline and (b) 0 01% SBI (soy bean enzyme inhibitor) — to show up clot aiding effects not due to the proteolytic enzyme To detect any traces of thrombin in the enzyme and thromboplastic reagents the proteolytic activity was inhibited with SBI and clotting tests run on a purified (prothrombin free) fibrinogen solution The presence or absence of fibrinolysis was noted in the plasma clots after eighteen hours (overnight) and in the clots which appeared in the fibrinogen When no clots were noted in the fibrinogen mixtures after two hours a strong (1000 u per ml.) thrombin was added Absence of clotting now denoted fibrinogenolysis If thrombin caused clotting the tubes were observed for lysis over another twenty four hours Finally the relative potencies of enzymes were assayed by the lysis of standard fibrinogen thrombin clots and the effect of the SBI on this was also noted The data summarized in Table XV permit the following conclusions

Thromboplastic aiding of the clotting of hemophilic plasma was most marked with tissue thromboplastin (essentially giving a normal "prothrombin clotting time") very good with cephalin and weak with platelet extract No proteolytic activity was detectible in the platelet or thromboplastic preparations All the enzyme preparations speeded up clotting also Dog lysin II was too strong and fibrino

TABLE XV

Clotting and Lysis Tests
On Recalcified Canine Hemophilic Plasma *(Citrated)*

	Agent Added	Clotting Times (37 C)		Clotting (37 C) of Fibrinogen + SBI	Fibrinolysis Times (37 C)	
		(1) Saline	(2) SBI (0.01%)		Saline	SBI
1	Saline	41 min (O)	41 min (O)			
2	Tpln	9.9 sec (O)	9.4 sec (O)	Neg *	Neg	Neg
3	Ceph (0.1%)	94 sec (O)	133 sec (O)	Neg *	Neg	Neg
4	Cryst Tryp (0.0025%)	155 sec (O)	42 min (O)	Neg *(O)	27 min	Neg
5	Comm Tryp	110 sec (L)	1 hr (O)	∞ (F)	190 sec	41 min
6	Dog lysin I	9¼ min (O)	26¼ min (O)	Neg *(O)	150 sec	Neg
7	" II	∞ (F)	10 min (L)	40 min (L)	45 sec	> 1 hr
8	" " III	14¾ min (L)	36 min (O)	140 min (L)	270 sec	> 4 hr
9	Bovine lysin	260 sec (O)	16½ min (O)	∞ (F)	270 sec	4 + hr
11	Saline	17¼ min (O)	45 min (O)			
10	Plat Extr	10½ min (O)	14¾ min (O)	Neg	Neg	Neg

(O) = No clot lysis overnight
(L) = Clot lysis
∞ (F) = Fibrinogenolysis (proved by adding 1,000 units per ml thrombin)
Neg * = Clots formed when tested 2 hours later with 1,000 units per ml thrombin and data on lysis of these clots in (parentheses)

genolysis interfered but the clot acceleration was seen in the presence of an enzyme inhibitor (SBI) The soybean inhibitor is not the best choice of antiprotease since it has some antithromboplastic action (seen in the cephalin experiment) but it is significant that it fails to abolish the clot aiding effects of the natural blood enzyme (lysin) preparations although it does inhibit completely the trypsin effect This difference between fibrinolysin and trypsin confirms earlier experiments on the activation of prothrombin in the presence of calcium A G and platelets(26) These facts render untenable the idea we(52) have long sought to substantiate namely a thromboplastic role for fibrinolysin analogous to that of trypsin They do however indicate that the enzyme preparations must contain some clot promoting factor the nature of which remains to be elucidated

REFERENCES

1 ANDREASSEN M *Haemofili i Danmark* København Munksgaard 1943

2 SKOLD E *On Haemophilia in Sweden and its Treatment by Blood Transfusion* Stockholm Norstedt 1944

3 FIELD R A RICKARD C G and HUTT F B Hemophilia in a family of dogs *Cornell Vet 36 285 (1946)*

4 GRAHAM J B *et al* Canine hemophilia observations on *course clotting anomaly and effect of blood transfusion J Exper Med* 90 *97 (1949)*

5 BRINKHOUS K M and GRAHAM J B Hemophilia in the female dog *Science* 111 *723 (1950)*

6 INTERNATIONAL CONGRESS OF HAEMOTOLOGY *Lancet* 2 *407 (1950)*

7 JURGENS R and FERLIN A Ueber den sog Prothrombinkonsumptionstest bei Hamophilie (Hamophile Konduktorinnen) und bei konstitutioneller Thrombopathie (v Willebrand Jurgens) *Schwei med Wchnschr* 80 *1098 (1950)*

8 LANGDELL R D GRAHAM J B and BRINKHOUS K M Prothrombin utilization during clotting comparison of results with the two stage and one stage methods *Proc Soc Exper Biol & Med 74 424 (1950)*

9 *BRINKHOUS K M Study of clotting defect in hemophilia* delayed formation of thrombin *Am J M Sc* 198 *509 (1939)*

10 ——— Initiation and acceleration factors in thrombosis *Blood Clotting and Allied Problems* Flynn J E Editor Trans First Conf New York Josiah Macy Jr Foundation 1948 (p 39)

11 BOGART R and MUHRER M E Inheritance of hemophilia like condition in swine *J Hered* 33 *59 (1942)*

12 MANTEUFFEL Z Bemerkungen zur Blutstillung bei Hamophilie Deutsch med Wchnschr 19 665 (1893)

13 SAHLI H Weitere Beitrage zur Lehre von der Hamophile Deut Arch f klin Med 99 518 (1910)

14 QUICK A J STANLEY BROWN M and BANCROFT F W Study of coagulation defect in hemophilia and in jaundice Am J M Sc 190 501 (1935)

15 BENDEIN W M and VAN CREVELD S On some factors of blood coagulation especially with regard to problem of hemo philia Acta med Scandinav 99 12 (1939)

16 PATEK A J JR and STETSON R P Hemophilia abnormal coagulation of blood and its relation to blood platelets J Clin Investigation 15 531 (1936)

17 PATEK A J JR and TAYLOR F H L Hemophilia some properties of substance obtained from normal human plasma effective in accelerating coagulation of hemophilic blood J Clin Investigation 16 113 (1937)

18 POHLE F J and TAYLOR F H L Coagulation defect in hemophilia Effect in hemophilia of intramuscular administration of globulin substance derived from normal human plasma J Clin Investigation 16 741 (1937)

19 LENGGENHAGER K Die Losung des hamophilen Blutungs und Gerinnungsratsels Mitt a d Grenzgeb d Med Chir 44 425 (1936)

20 BRINKHOUS K M Study of clotting defect in hemophilia delayed formation of thrombin Am J M Sc 198, 509 (1939)

21 ——— Clotting defect in hemophilia deficiency in a plasma factor required for thromboplastin liberation from platelets Federation Proc 6 389 (1947)

22 QUICK A J Quantitative studies on the coagulation defect in hemophilia Federation Proc 6 284 (1947)

23 BRINKHOUS K M Clotting defect in hemophilia deficiency in plasma factor required for platelet utilization Proc Soc Exper Biol & Med 66 117 (1947)

24 QUICK, A J Studies on enigma of hemostatic dysfunction of hemophilia Am J Sc 214 272 (1947)

25 STEFANINI M and CROSBY W H Complementary correction of the defective coagulation mechanism of hemophilic and thrombocytopenic blood Proc Soc Exper Biol & Med 73 301 (1950)

26 QUICK A J The present status of platelets in blood coagula tion Blood Clotting and Allied Problems Flynn J E Editor Trans Second Conf New York Josiah Macy Jr Foundation 1949 (p 201)

27 TOCANTINS L M CARROLL R T and HOLBURN R H Influence of actual plasma concentration in the final clotting mix ture on the response of normal and hemophilic plasmas to thromboplastin Implications in regard to one stage prothrom

bin time tests *Blood Clotting and Allied Problems* Flynn J E Editor Trans Third Conf New York Josiah Macy Jr Foundation 1950 (p 192)

28 FONIO A Beobachtungen uber den Gerinnungs—und den Thrombosevorgang im Dunkelfeldnativpraparat *Schweiz med Wchnschr* 21 *510 (1940)*

29 ZATTI P Sulle cause dell agglutinazione delle piastrine nel sangue in via di coagulazione *Boll Soc ital biol sper* 24 22 *(1948)*

30 WRIGHT J H and MINOT G R The viscous metamorphosis of the blood platelets *J Exper Med* 26 *395 (1917)*

31 PINNINGER, J L and PRUNTY F T G Some observations on bloodclotting mechanism role of fibrinogen and platelets with reference to case of congenital afibrinogenaemia *Brit J Exper Path* 27 *200 (1946)*

32 KAY J H Zinc precipitation of plasmin *Science* 112 *225 (1950)*

33 SCHALES O *et al* Inhibition of enzymatic proteolysis I Observations with carbonyl group reagents effect of hydrazine on peptic hydrolysis *Arch Biochem* 19 *119 (1948)*

34 GUEST M M and WARE A G Dissolution of fibrin clots in the presence of concentrated purified thrombin *Federation Proc* 9 *53 (1950)*

35 BORDET J and DELANGE L Analyse et synthese du processus de la coagulation *Ann et bull Soc roy d sc med et nat de Bruxelles* 72 *87 (1914)*

36 FANTL, P and NANCE M H Acquired haemorrhagic disease in female due to inhibitor of blood coagulation *M J Australia* 2 *125 (1946)*

37 TONCANTINS L M CARROLL R T and MCBRIDE T J A lipid anticoagulant from brain tissue Physiochemical characteristics and action *in vitro* and *in vivo* *Proc Soc Exper Biol & Med* 68 *110 (1948)*

38 TOCANTINS L M CARROLL R T and HOLBURN R H The clot accelerating effect of dilution on normal and hemophilic blood and plasma Relation to the mechanism of blood coagulation *Blood* (in press)

39 MILLS C A Blood clotting studies in hemophilia *Am J Physiol* 76 *632 (1926)*

40 TOCANTINS L M and CARROLL R T Activity curves of crude and purified inhibitors and accelerators of blood coagulation *Proc Soc Exper Biol & Med* 69 *431 (1948)*

41 WARE A G and SEEGERS W H Two stage procedure for the quantitative determination of prothrombin concentration *Am J Clin Path* 19 *471 (1949)*

42 ——— Plasma accelerator globulin partial purification quantitative determination and properties *J Biol Chem* 172 *699 (1948)*

43 LANGDELL R D GRAHAM J B and BRINKHOUS K M Prothrombin utilization during clotting comparison of results with the two stage and one stage methods *Proc Soc Exper Biol & Med 74 424 (1950)*

44 POHLE F J and TAYLOR F H L Coagulation defect in hemophilia Effect in hemophilia of intramuscular administration of globulin substance derived from normal human plasma *J Clin Investigation 16 741 (1937)*

45 SINGER K *et al* Circulating anticoagulants in hemophilia and in hemophilia like disease *Blood 5 1135 (1950)*

46 FROMEYER W B EPSTEIN R D and TAYLOR F H L Refractoriness in hemophilia to coagulation promoting agents whole blood and plasma derivatives *Blood 5 401 (1950)*

47 TOCANTINS L M and CARROLL R T Separation and assay of a lipid antithromboplastin from human brain blood plasma and plasma fractions *Blood Clotting and Allied Problems* Flynn J E Editor Trans Second Conf New York Josiah Macy Jr Foundation 1949 (p 11)

48 ——— Distribution and assay of inhibitors and accelerators of blood coagulation in extracts of organs of normal and hemophilic man *Federation Proc 9 127 (1950)*

49 HILL J M *et al* Problems in hemophilia Presented before the Third Mexican Congress of Transfusion and Hematology Nov 22 1950 Mexico D F (unpublished)

50 HALDANE J B S Rate of spontaneous mutation of human gene *J Genetics 31 317 (1935)*

51 GATES R R *Human Genetics* New York The Macmillan Co 1946 Vol 1 (pp 581 625)

52 FERGUSON J H TRAVIS B L and GERHEIM E B Activation of prothrombin with special reference to thromboplastic enzyme (tryptose) *Blood 3 1130 (1948)*

CHARACTERISTICS OF BLOOD PLATELETS THEIR SIGNIFICANCE IN THROMBUS FORMATION

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THE RECOGNITION OF the blood platelets in the middle of the last century by Zahn(1) together with concurrent improvements in microscopy effected a radical change of ideas on the relation of thrombosis to clotting Bizzozero(2) was probably the first to appreciate the distinction between these two during the course of his observations on the deposition of platelets at sites of damage in vessel walls In particular he noted that a true thrombus only forms where platelets have already aggregated

A few years later Eberth and Schimmelbusch(3) published their classical monograph in which they described observations on trans illuminated blood vessels in animal mesenteries in which white thrombi could be caused to form experimentally by the application of pressure heat or chemicals to their walls They watched the gradual blocking of the vascular lumen by the agglutinated platelets and the consequent stagnation of blood cells both before and behind the occlusion and finally the coagulation of this column of blood to form a red clot

These pioneer experiments have been repeatedly confirmed and the use of microcinematography such as in the excellent film which was recently produced by Hoffman La Roche at Basle has further clarified some of the finer details of the formation of white thrombi in injured mammalian vessels that take place too rapidly to be followed by the observer s eye The first recognizable change is the piling up of platelets which adhere together to form a glistening granular plaque over the traumatized point

The rate at which this occurs depends mainly on two factors First the number of platelets available and the second their ad hesiveness The numbers may be effected in two ways – either by alterations in the actual count in the blood or by changes in the rate at which the blood is passing by for obviously when the count

is constant more platelets are available in a fast stream than in a comparatively slow one But should the count be raised greater numbers of platelets may pass through a vessel even though the velocity of the flow may be somewhat slower

As the white thrombus grows it encroaches progressively on the lumen of the vessel though the passing stream can often be seen to break off small clumps of the aggregated platelets and carry them away from the parent mass In spite of these alterations of erosion and deposition the plaque constantly enlarges in a way that Aschoff(4) has compared to the continually shifting but gradual silting up of sand near a breakwater

As the patency of a vessel is reduced the rate of flow of the blood behind and in front of the obstruction falls off until when occlusion is complete the stage is set for the propagation of the clot by the coagulation process

The disintegrated platelets liberate their active principal and coagulation of a column of stagnant blood takes place between the site of injury and the anastomosis up and downstream As neighboring vascular junctions however the advancing red coagulum again comes into contact with actively flowing blood

One of two sequelae may result either the local rate of flow is so rapid that any platelets deposited will be washed clear again in which case the endothelial lining of the vessel migrates over and seals over the bare end of the clot so that the process is arrested or if the velocity of the blood current is less such platelets will remain adherent and the clot propagates into the larger vessel In this latter case the process of white thrombus formation followed by occlusion stagnation and coagulation is repeated and this cycle may recur from one tributary branch to another until such large vessels as the femoral veins may in time be involved

Recently Dr Quick(5) has recapitulated this process and has drawn attention to a significant feature worthy of attention Intimately associated with the primary clot and dependent upon the liberation of kinase from its disintegrating platelets, a layer of coagulum is formed the retraction of which expresses serum This serum promotes the further deposition of platelets and this accounts through the successive repetitions of the cycle for the alternate red and white components of a typical mixed thrombus

From this brief summary of the process of thrombosis it is evident that some of the major problems that present themselves for

solution are concerned with the factors that influence the inherent adhesiveness of the platelets It was in an attempt to measure this property and to give it a quantitative expression that I originally devised the simple technique of subjecting a blood sample rendered noncoagulable with a minimal amount of heparin to slow rotation in a glass bulb tube

Figure 37 shows the primitive apparatus that I used It is a plain wooden wheel with the tubes at right angles to it driven by a little motor(6) The type of tube used is shown at the base

Determinations of successive platelet counts in samples taken at regular intervals provided a direct measure of adhesiveness and by expressing these counts as percentages of these initially found and plotting them against the time comparative curves for the sticki ness of the platelets under various conditions could be made

Figure 38 shows the original curves for heparin there are three different concentrations The plottings marked "one" were the smallest concentration of heparin those marked "two" were a slightly higher concentration and "three" was the greatest concen tration I used I separated the samples from the males and females to show that there was no sex difference and the top line "C" is a control tube that was lined with vaseline

Figure 39 shows the other anticoagulants that I tried at that time sodium oxalate in three concentrations and chlorazol I also tried chlorazol blue dye and I always obtained agglutination of platelets and threw it out(6) All of the different anticoagulants which were tried — heparin oxalate and chlorazol dyes *in vitro* and dicumarol *in vivo* reduced the stickiness of the platelets when compared with the reference sample

As I showed in the first figure with heparin the stickiness varied inversely with the concentration of the anticoagulant and from these results it appeared that the adhesiveness was associated in some way with the coagulation and is affected by anticoagulant drugs irrespective of where they exert their action

The control tubes which were lined with a thin layer of vaseline were always rotated at the same time to determine the number of platelets which were lost by disintegration Such losses never ex ceeded more than 10 per cent by the end of eighty minutes In the early work also I did red blood counts at each ten minute interval to see that the mixing of the sample was maintained

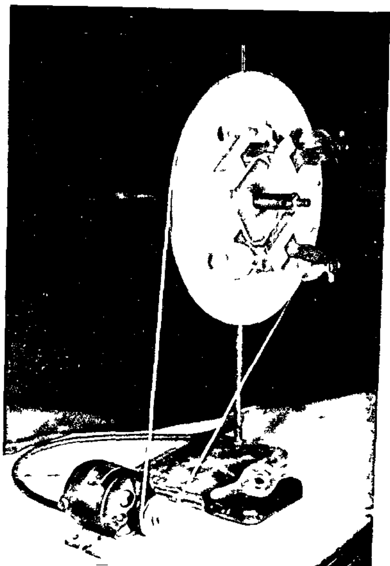


FIGURE 37

Now since interference with the coagulation mechanism appeared to reduce the adhesiveness of the platelets I went on to see whether under certain circumstances which favored clotting their stickiness was enhanced

It has long been known that in the postoperative and puerperal periods the circulating platelet count first rises sometimes to twice or thrice its initial value and then falls and that thrombosis fre

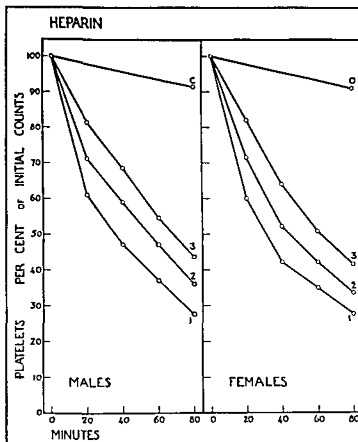


FIGURE 38 Decrease of platelet numbers expressed as percentage of initial counts Curve C Control
1—lowest concentration of heparin
2—intermediate concentration
3—highest concentration

quently occurs about the time the maxim is reached Was there at that time any change in their adhesiveness? It was known that their agglutinability was increased in the posttraumatic period but no measure of stickiness for other types of surface was available

By making determinations of the variations in this property that developed on the different days after operation or parturition it was possible to show that the curves for adhesiveness as well as

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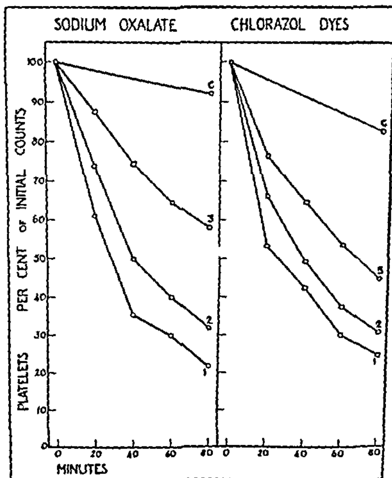


FIGURE 39 Decrease of platelet numbers expressed as percentage of initial counts

the counts passed through a maximum(7) Figure 40 shows the stickiness curves on various days following operation and delivery On the day before operation, in every case the stickiness was normal But in the subsequent days the stickiness of the platelets showed a progressive increase Since the count was highest about the tenth day it seemed probable that the increased stickiness was dependent upon the outpouring of new platelets from the bone marrow into the circulation in response to the stimulation of trauma

In order to test this idea experimentally a raised platelet count was induced in rabbits by two different methods(8 9) which are believed to depend on different underlying mechanisms In the first

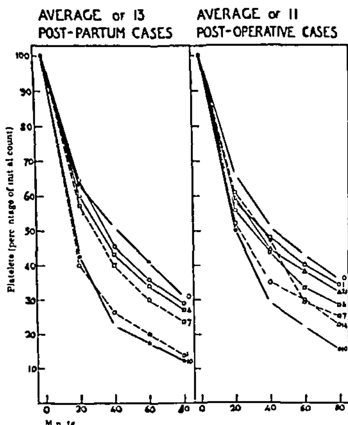


FIGURE 40 Number of platelets expressed as a percentage of this initial count. Curves are numbered to correspond with the day of observation Reprinted by permission from Wright H P *J Path & Bact* 54 461 (1942) (8)

adrenalin was injected this drug is known to cause the prompt liberation of mature platelets from depots in the lungs and spleen Stickiness curves were obtained at the peak of the response and these did not differ significantly from those found in prior tests on the same animals In the second pyridine was injected intravenously in small doses to stimulate the production of new platelets by megakaryocytes in the bone marrow The peak of the count occurred about four hours later When stickiness curves were made under these conditions it was found that the platelets showed an augmented adhesiveness very similar quantitatively to that found in surgical and puerperal cases

Finally a splenectomy was performed on each animal an operation which raises the platelet count markedly On the various days following this operation platelet counts and stickiness curves were made pyridine was then injected, and the counts and curves repeated four hours later

Figure 41 shows how in the days following the splenectomy the counts and stickiness were progressively less altered by the injection of pyridine It seemed that when the bone marrow had been fully stimulated by the products of trauma it was unable to respond further to the injection of pyridine

These observations can best be determined on the supposition that the posttraumatic increase both in counts and stickiness are dependent on the new outpouring of platelets rather than upon the mere mobilization of mature ones from other sites in the body

Seven days after operation when the platelet count was highest the pyridine had practically no effect In other words they were already fully sticky as a result of the trauma

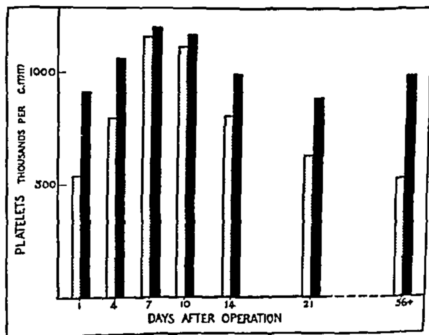


FIGURE 41 Average platelet count before and four hours after the injection of pyridine following splenectomy *Grey* — Average "zero" platelet count in thousands per c mm *Black* — Average platelet count after pyridine injection Reprinted by permission from Wright H P *J Path & Bact 53 151 (1941)* (9)

Many of these observations on platelets have now been repeated by other workers Murray Weiner Shapiro and their colleagues(10) have confirmed a number of these earlier findings and have investigated more types of hematological conditions than I had at that time available

Spooner and Meyer(11) have repeated the observations on the effect of dicoumarin in reducing platelet adhesiveness They did not find any decreased stickiness Recently Moolten and his associates(12) have evolved a new technique for measuring platelet stickiness which obviates one of the main criticisms of my method namely that the eighty minute period of observation may allow the disintegration of the platelets to contribute too much to the removal of these blood elements from the sample These workers agreed that stickiness is enhanced in the postoperative period but they did not find the decreased stickiness which I found to be a feature of hemophilia(13) They also concluded that platelet adhesiveness was little affected in the dicoumarized subject unless the prothrombin level had been lowered for more than two days All my rabbits on which the first observations had been made had received this drug from a week to a fortnight so that their prothrombin levels were well depressed

Accepting therefore that under differing conditions platelets exhibit variations in their ability to adhere to foreign surfaces I think it might be profitable to discuss briefly the physicochemical and mechanical conditions which may affect this property Normally the suspension stability of all the constituent formed elements of the blood is extremely high but it has been known since the days of Osler s(14) study of platelets in 1886 that it can be very easily upset in normal blood

The factors which appear to maintain the various elements in suspension are first their surface charges which are of the same sign and second their constant motion both as a mass and relative to one another so that even when the elements approach each other closely they tend to separate again for purely mechanical reasons It is believed moreover that the surfaces of the formed blood elements carry variable amounts of adsorbed water and that this helps to render them stable in suspension as well as augmenting the viscosity of the blood

The electrokinetic potential(15) and the cataphoretic velocity(16) of the platelets have been found to be similar to those of neutro

philes which suggests that most of these types of cell ordinarily have the same surface characteristics Such findings have led perhaps mistakenly to the belief that the *platelet behaves purely* passively as far as its surface is concerned and that the nature of its exterior is entirely dependent upon the nature of the surrounding medium

For example *according to Govaerts(17), washed platelets resus* pended in saline do not exhibit characteristic adhesiveness With the platelets in plasma of normal composition however it seems likely that their surface reactions may be in part dependent on some of the metabolic activities of the cell and in particular on the seeping of small amounts of the active principal through its membrane to bring about an almost monomolecular layer reaction of the coagulation process on its outer surface This view has also been proposed by Copley(18) on the basis of his own observation and that of Enders and Herget(19) that the surface of platelets is more easily penetrated by ions than is that of the red cell envelope

If the products of platelet activity escape through the surface the cell plasma interface would become covered with a film of fibrin Stickiness may be envisaged qualitatively as the property of two surfaces after coming into contact to resist separation and in this general sense such a fibrin film would render the platelet surface sticky Moreover where the viscosity of such a surface contact film is great as with fibrin the mechanical shearing force required *to separate two adherent particles is high and even though they* may carry a like electric charge their mutual repulsion may not suffice to overcome the cementing action of the viscous surface film

In view of the frequency of thrombosis in the postoperative period *it is important to recognize certain characteristic changes* affecting the blood which tend to lower its stability as a suspension It was shown by Fahraeus(20) that the addition of hydrophilic colloids reduced the suspension stability of the erythrocytes and the same was later demonstrated for the leukocytes by Vejlens(21) These hydrophilic colloids act by reducing the surface charges on the cells and by modifying the positions of the adsorbed water on the cell surface The cells are thus enabled to approach each other more closely and because their electrokinetic potentials are reduced their surface tension is sufficiently great to overcome their mutual repulsion so that aggregation takes place Of the hydrophilic colloids studied fibrinogen and globulin are the most potent in producing

this change in surface character and it is possible that the increased tendency for platelets to stick together (which is characteristic of of the postoperative period) may be partly due to the increase of these substances in the plasma

Although the present discussion is mainly on thrombosis and coagulation it would be a pity to omit any reference to the important part taken by platelets in host resistance to invading micro organisms especially because the analogy may be pertinent Phenomena of platelet loading in which the platelets adhere to circulating bacteria was investigated in the first two decades of the present century by Levaditi(22) Aynaud(23) Govaerts(24) and Roskam(25 26)

It was found by Govaerts that plasma or serum was necessary for this heterogeneous aggregation to take place and he believed it was due to a surface effect caused by a substance in the suspending medium Roskam regarded it as analagous with opsonization" the deposition on the bacterial surface of a protein film which facilitates phagocytosis by host cells

An interesting example of platelet loading is the Reichenberg Reaction" in experimental trypansomiasis In this reaction these parasites when introduced into the bloodstream of immune animals become heavily coated with platelets while in the unimmune animal no such loading occurs

Quite recently the observations of Copley and Houlihan(27) have suggested that the clumping of the platelets with bacteria is increased to some degree unspecifically by the addition of plasma but so far as I know they have not published any findings on the effects of specific antisera on this phenomenon It is interesting in this connection that immune bodies are mainly if not wholly limited to the globulin fractions of the plasma and may therefore exert a particularly marked action on such a surface phenomenon

There is a further factor which is closely connected with those already considered but must find a place in any general review Should the surface charge on the platelet be reduced by changes in the plasma it becomes possible for it to come into closer proximity both with other blood elements or with the blood vessel wall

The normal intima has been regarded as unwettable though the interpretation of this term in clear physical concepts is still obscure Wettability" and nonwettability may be dependent upon the

surface potential of some boundary layer but it may also be due to the type of hydrocarbon present in the interface It is likely that that the intima cell membranes are composed of a protein lipoid network which is devoid of hydrophilic properties It is only when the integrity of the cell membrane of the intimal endothelium is broken as by trauma that the platelets are able to adhere to the vessel walls and initiate a thrombus At any lesion in the intima a wettable point is produced by the disruption of this lipoidal membrane and since the endothelium also carries a surface charge damage to these cells may alter the potential relative to that on the platelets and thus facilitate their local deposition

The old experiments in which one vein is tied off gently while another is tied off and then injured with a needle both being open at the end of twenty four hours illustrates this point In the uninjured vessel the blood remains fluid while in the injured one a complete clot is formed though adherent to the wall only at the point of trauma

The importance of unwettability of the surface with which the blood comes in contact has moreover been demonstrated practically by Dr Jaques(28) in his experiments on the behavior of platelets in silicone glassware When the platelets come in contact with a wettable surface the spreading phenomenon is observed and the disintegration begins The lytic change liberates the active principal fibrin formation is promoted and the surface of the aggregated platelets is rendered still more sticky

Should this hypothesis of surface changes and especially that of the production of surface films of fibrin be true it would explain the reduction of platelet stickiness which has been observed in the presence of all types of anticoagulants These substances interfere with the formation of fibrin at some critical stage of production so that the surface film would possess less viscosity It would also explain why washed platelets suspended in saline lack adhesiveness because in this medium there can be no production of fibrin

Finally for young platelets it seems reasonable to assume either that their cell membrane is more permeable or that their active principle is more potent and therefore capable of causing the deposition of more fibrin on their surface

Having reviewed briefly some of the physicochemical changes which influenced the deposition of platelets I will conclude by saying a few words on the importance of hemodynamics in formation of venous thrombi

Normally in small veins axial flow is brought about by the viscous and particulate nature of blood and ensures that the formed elements are separated by a zone of the plasma from the intimal walls This axial flow acts centripetally on the corpuscular constituents of the blood so that the white cells which are the largest move in the center surrounded by the somewhat smaller red cells Tending to lie externally still are the blood platelets which because of their smaller size are forced out into the peripheral fluid laminae But although in the outer part of the corpuscular stream they do not normally come into contact with the intima from which they are separated by the plasmatic layer They are however the first to enter this layer should any slowing of the blood stream reduce the centripetal force of the axial flow Moreover should conditions favor their deposition on the vessel wall they are in that layer in which any tendency to separate them or dislodge them from the mural endothelium by shearing stresses are minimal In both these ways therefore any reduction of flow rate may be expected to facilitate platelet deposition and the formation of thrombi

Unfortunately few studies of the factors that affect venous blood flow rates in limbs have yet been made for until radioisotopes became available no satisfactory methods had been devised Many valuable observations on flow rate over long circulatory paths have been made but for obvious reasons they are unsatisfactory for recognition of stasis in the lower limbs

In the last three years we have been studying alterations in flow rate of venous blood in both upper and lower limbs An injection of a small volume of isotonic radiosaline is made at the ankle or on the dorsum of the foot for leg measurement and at the wrist or on the back of the hand for those of the arm and the passage of the isotope as it passes the groin or axilla is recorded by a screened Geiger Muller counter(29)

Figure 42 shows the type of apparatus that we have been using It is a good photograph of the feet The screened counter is easily moved in all directions and is arranged over the femoral triangle in the groin The central unit in the apparatus is the ordinary scaling unit used for assay work and the lower section is the main stabilizer It works in such a way that every impulse from the Geiger Muller counter brings the pen mechanism — which cannot be seen too well in this picture — down one notch so that a kymograph tracing of the count is recorded

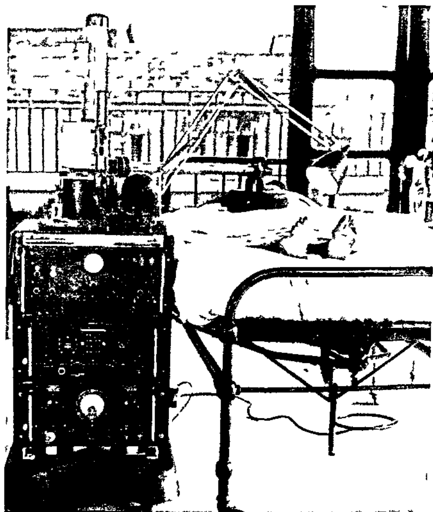


FIGURE 42 Apparatus used for venous blood estimation

Figure 43 shows a portable apparatus which is an improvement on that shown in Figure 42 The Geiger counter is screened by lead on a tripod which can be used for the groin by fitting one leg of the tripod between the patient's leg and the other two at the side or it can be tipped up on end in the position of a dog begging so that it then comes into the axilla Instead of the kymograph being of the usual round type it is a straight paper strip The accuracy of the method is to about one second plus or minus

The kymograph tracing is quite clear and on it is recorded manually the moment of injection the moment at which the injected

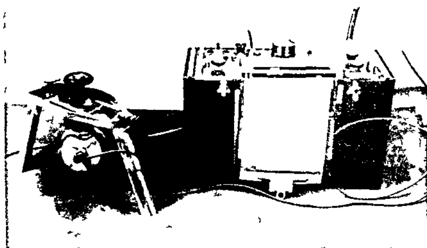


FIGURE 43 Portable apparatus used for venous blood flow estimations

solution reaches the counter by the change of slope obtained on the kymograph and the time marker Then by measuring the distance from the recorded moment of the injection one can obtain the mean venous flow rate

We have not completed these observations We had to begin by measuring large numbers of normal persons (30) in order to get the necessary basic data and I will not go into details of these findings here Suffice it to say that during pregnancy there is a progressive slowing of the flow in the leg which becomes more evident when the fetal head engages and still more so throughout labor These are the average figures from a group of eighty six women(31) Figure 44 shows the actual findings the grey area being plus or minus three times the standard error The ordinate is expressed in time in seconds (not in flow rate) while the abscissa represent the weeks of gestation There is a steady slowing of the blood flow in the leg which reaches its peak when labor commences After delivery it returns rapidly to normal The engagement of the fetal head also retards the flow significantly though it is masked in this figure based on average figures for the group The head descends at any time between the twenty eighth week of gestation and the first stage of labor so the individual changes are not demonstrable in such a chart as this We think the slowing

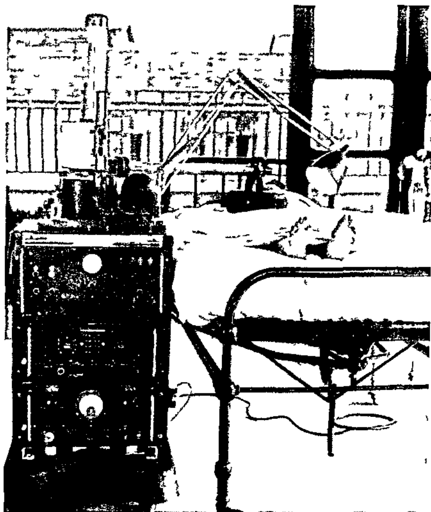


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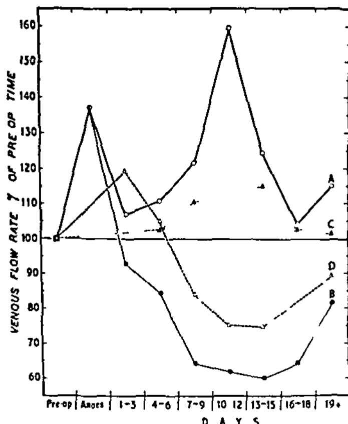


FIGURE 45 Average rates of venous flow on various days after operation expressed as percentages of the preoperative flow rate

Curve A — Ambulant cases legs
C — Ambulant cases arms
D — Nonambulant cases arms
B — Nonambulant cases legs

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In those patients who were confined to bed there is a general circulatory slowing in the postoperative period which is most pronounced *about the tenth day In all cases the reduction of flow is* far more marked in the legs than in the arms In the group who had been allowed early ambulation on the other hand not only was there no slowing in either limb but an increase in rate during the critical period Without laboring the point this finding clearly upholds the old view that stagnation occurs postoperatively and may well contribute to the onset of posttraumatic thrombosis Such

is brought about mechanically by the increased intra abdominal pressure We are fairly certain of this as we also investigated cases of ascites in whom the intra abdominal pressure was likely to be comparable These cases showed a slow flow while their bellies contained fluid but immediately after paracentesis when the intra abdominal pressure was reduced, it returned to normal

When we published these observations we suggested that the reason why more women do not develop thrombosis during the puerperium is because the stagnation factor is relieved before the typical puerperal cytological and chemical changes in the blood have occurred

More recently we have studied two large groups of surgical cases one of which was composed of ambulatory cases and the other of those confined to bed(32) The rate of flow in arms is 10 cm per second in the initial observations compared with that of 4 5 cm per second in the legs Figure 45 therefore expresses the preoperative observations respectively for arms and legs as 100 per cent A truer picture of the relevant changes is presented in this way

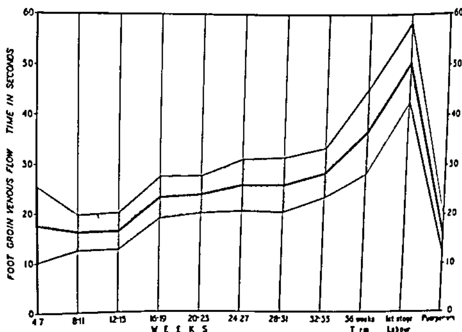


FIGURE 44 Average flow times in the legs of 61 women at each month of pregnancy in labor and the puerperium Shaded area = ± standard error x3 Reprinted by permission from *Surg Gynec & Obstet 90 481 (1950)* (32)

one hand and toward the physicochemical factors of the platelets on the other

Zucker The same postoperative increase in adhesiveness was observed in animals was it not? And the animals were certainly not kept in bed

H P Wright No they certainly were not kept in bed

Barker Do you feel that there has been a reduction of the inci dence of postoperative thrombosis as the result of early ambulation?

H P Wright Well there is a reduction of incidence of severe thrombosis

Barker Do you have statistical evidence to show that?

H P Wright Not from my own hospital but the figures that have been published mostly in this country do suggest that early ambulation has reduced it but not eliminated it

Barker There seems to be a difference of opinion about that in different clinics As far as we can determine at the Mayo Clinic there has not been a reduction in incidence of postoperative throm bosis as the result of early ambulation

Kay The data from Charity Hospital where early ambulation and other prophylactic measures have been practiced for some time show a yearly increase in both thrombosis and embolism on all services in the period 1938 to 1949(33)

Link I am wondering whether that might not be due to the fact that the physicians are now loading people with drugs penicillin etc

H P Wright But you haven t kept the count of the number of intravenous injections infusions and everything else that goes into the veins I think if you could parallel those you might find that the increase or at least shall we say the lack of decrease is prob ably due to that

Barker That is an assumption which nobody has proved as yet There are good reasons for early ambulation following operations from the standpoint of preventing some postoperative complica tions but I do not think that it has been shown conclusively that early ambulation as it is practiced has influenced the incidence of postoperative thrombosis

a fall in flow rate reduces both the centripetal force on the blood elements and the shearing stress to which any plaque of adherent platelets is subjected to

DISCUSSION

Tocantins In ambulatory patients how long would you have them rest before you make the determination?

H P Wright Our determinations have been made under standard conditions of temperature rest and so on Of course in regard to the bed patient the condition of rest is not steady but in the ambulatory cases the same standard conditions are imposed as in our normal series they are kept quiet for a half hour under standard temperature conditions to make sure of that

Knisely Have you ever measured one of those just walking?

H P Wright I have but I do not have much data on that I have been resting them for the standard time then making a determination Immediately after the first measurement I have had them moving their feet and legs vigorously though still in the horizontal position A second determination after this always shows a markedly increased rate of flow

Brambel How about platelet adhesiveness during early ambulation? Have you done any studies that would be comparable to these?

H P Wright I have not attempted to do that

Knisely You should

H P Wright I do not see why early ambulation should have anything to do with adhesiveness of platelets It doesn t click with me

Brambel Except that you are showing more rapid circulation

H P Wright Well adhesiveness is dependent upon the platelets and not upon the circulation Really the two aspects of the work which I have attempted to put before you are to my mind distinct Both however are pertinent to the clinical finding that the majority of posttraumatic thrombi are diagnosed on about the tenth day These researches of mine have been directed toward elucidating the hemodynamics of the postoperative period on the

Warner I would like to ask Dr Kay if he has any data on the age incidence of patients in the hospital We had that same experience with two series of postmortem cases ten years apart One was prior to use of early ambulation and the other ten years later after early ambulation had been the practice for several years There was actually a slight increase in the incidence of thromboembolism in cases coming to autopsy in the second group During this same ten year period we had had an increase of approximately ten years in the average age of the patients The average age had gone up from fifty six to about sixty six We were steadily getting into an older and older group of patients which well might offset and mask any slightly beneficial effect of early ambulation for all we could tell There was this rather marked change in the average age of the patients with whom we were dealing

I S Wright That s why an alternate case series would be of benefit

Kay The figures have been published(36) They were broken down for age sex race seasonal and hospital service incidence as well as for the incidence according to associated conditions and site of involvement All the data tend to confirm the concept that the incidence of thromboembolism is increasing

I did not mean to suggest that early ambulation should be abandoned I would be the last to suggest that patients be kept at constant bed rest after operation Indeed in the work we have done on predicting thrombosis this process has occurred with more prolonged prothrombin times when patients were not ambulated

We have not had very many patients who were not ambulated However it is true that some of the older patients after major surgical procedures cannot be ambulated You can lift the patient out of bed and carry him around it but you cannot make him walk Such a procedure may reduce the incidence of thrombosis in the surgeon s leg but I doubt that it affects the incidence in the patient s leg

I S Wright I think we will ask Dr Zucker to discuss her presentation

Brambel We could name the surgeon on the basis of the fibrinogen determination postoperatively

Kay In our work the patient has been considerably more important in the response to surgery than has the operator Some

I S Wright This is a perfect example of where a well controlled case series would have given a definite answer Many surgical anesthetic and medical techniques have been modified during recent years so that no figures comparing the present with the past can be considered valid as far as conclusions regarding any single factor may be concerned

Link And the bread that we use contains substances which keeps substances fresh — so called pliable great big molecules Rats refuse bread

H P Wright I entirely agree with you Dr Barker I am not suggesting that this is the answer to thrombosis but I am trying to point out that the stagnation factor exists a point which we have never really known before In other observations this point has not been clear as the measurements have been taken over the lesser circulation as well as the greater

Quick I think early ambulation ought to be defined too In many instances it merely means that the patient is supposed to get out of bed walk over to a window and sit on a chair with his legs hanging down

H P Wright And that is a chair that catches him underneath the knees Do you know Simpson s work(34) on the pulmonary embolism?

Well that s the sort of thing that happens in the wards They go and sit on a hard chair

Barker I think that is a very important point Most patients who have just had extensive abdominal operations — the types of operation which are most frequently complicated by thrombosis — do not ambulate or exercise their legs even when they are urged The process is too painful

Jaques Another point also to consider in the large number of clinical series cited as statistical evidence is the great variation in the operator This is one of the most important variables and one that is completely forgotten Roskam and Fontaine(35) quote Leriche to the effect that a great variation in the incidence of thrombosis exists on the basis of the actual surgeon operating This factor is mixed up because there are a number of different operators in the same hospital contributing to the series It is very difficult to say just how the control and the experimental series are comparable or whether they are not comparable

Warner I would like to ask Dr Kay if he has any data on the age incidence of patients in the hospital We had that same experience with two series of postmortem cases ten years apart One was prior to use of early ambulation and the other ten years later after early ambulation had been the practice for several years There was actually a slight increase in the incidence of thromboembolism in cases coming to autopsy in the second group During this same ten year period we had had an increase of approximately ten years in the average age of the patients The average age had gone up from fifty six to about sixty six We were steadily getting into an older and older group of patients which well might offset and mask any slightly beneficial effect of early ambulation for all we could tell There was thus rather marked change in the average age of the patients with whom we were dealing

I S Wright That's why an alternate case series would be of benefit

Kay The figures have been published (36) They were broken down for age sex race seasonal and hospital service incidence as well as for the incidence according to associated conditions and site of involvement All the data tend to confirm the concept that the incidence of thromboembolism is increasing

I did not mean to suggest that early ambulation should be abandoned I would be the last to suggest that patients be kept at constant bed rest after operation Indeed in the work we have done on predicting thrombosis this process has occurred with more prolonged prothrombin times when patients were not ambulated

We have not had very many patients who were not ambulated However it is true that some of the older patients after major surgical procedures cannot be ambulated You can lift the patient out of bed and carry him around it but you cannot make him walk Such a procedure may reduce the incidence of thrombosis in the surgeon's leg but I doubt that it affects the incidence in the patient's leg

I S Wright I think we will ask Dr Zucker to discuss her presentation

Brambel We could name the surgeon on the basis of the fibrinogen determination postoperatively

Kay In our work the patient has been considerably more important in the response to surgery than has the operator Some

patients go through a procedure without showing any fibrinogen B which as you know is a fair measurement of nonspecific injury

Seegers What was that?

Kay Fibrinogen B usually appears in response to tissue injury infection or trauma

Seegers I wasn t aware of that

Kay I am quoting Colonel Pulaski and Captain Voorhies(37) Our own work with fibrinogen B tends to confirm this correlation

H P Wright We have been trying to show the presence of fibrinogen B as Lyons has done

Kay Well we found no correlation with thrombosis

H P Wright Nor did we

Kay We did find some correlation with the amount of trauma

H P Wright We found that any surgical case if you followed him day by day showed an increase of fibrinogen B at about the time you would expect to diagnose a thrombus and on that basis you would treat every case

Kay In our experience seventy five of eighty seven patients developed fibrinogen B at some time during the postoperative hospital stay On the other hand eight patients withstood major surgical procedures without developing any

H P Wright I suspect they wouldn t show other things as platelet differences and so on

Link Whose nomenclature is involved on fibrinogen B is that the Harvard?

Edsall No we have not used that terminology

REFERENCES

1 ZAHN F W Untersuchungen uber Thrombose Bildung der Thromben *Virchows Arch f Path Anat 62 81 (1875)*

2 BIZZOZERO J Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung *Virchows Arch f Path Anat 90 261 (1882)*

3 EBERTH J C and SCHIMMELBUSCH C Experimentelle Untersuchungen uber Thrombose *Virchows Arch f Path Anat 103 39 (1886)*

4 Aschoff L Thrombose und Embolie *Verhandl d Deutsch Gesellsch f Kreislaufforsch* 7, *11 (1934)*

5 Quick A J Clot retraction Its physiological and clinical significance *Am J Med Sc* 220 *538 (1950)*

6 Wright H P Adhesiveness of blood platelets in normal subjects with varying concentrations of anti coagulants *J Path & Bact* 53 *255 (1941)*

7 ——— Changes in adhesiveness of blood platelets following parturition and surgical operations *ibid* 54 *461 (1942)*

8 ——— Sources of blood platelets and their adhesiveness in experimental thrombocytosis *ibid* 56 *151 (1944)*

9 ——— Adhesiveness of blood platelets in rabbits treated with dicoumarol *ibid* 57 *382 (1945)*

10 Weiner M *et al* Platelet adhesiveness *Blood* 3 *1275 (1948)*

11 Spooner M and Meyer O O Effect of dicoumarol (3 3 methylenebis [4 hydroxy coumarin]) on platelet adhesiveness *Am J Physiol* 142 *279 (1944)*

12 Moolten S E and Vroman L Adhesiveness of blood platelets in thromboembolism and haemorrhagic disorders *Am J Clin Path* 19 *701 (1949)*

13 Wright H P Adhesiveness of blood platelets in haemophilia *Lancet* 1 *306 (1946)*

14 Osler W On certain problems in the physiology of blood corpuscles *Medical Classics* Baltimore Williams & Wilkins Co 1939 (Vol IV p 178)

15 Cremer H D Über das electrokinetische Potential der Thrombocyten *Broch Zeitsch* 281 *345 (1935)*

16 Abramson H A Electrophoresis of blood platelets of horse with reference to their origin and to thrombus formation *J Exper Med* 47 *677 (1928)*

17 Govaerts P Intervention of opsonins in the adhesion of bacteria to platelets *Compt Rend de Biol* 83 *196 (1920)*

18 Copley A L and Glaser D F Studies on platelets effects of platelets on surface tension of saline solutions and heparinized plasma *Blood Spec Issue* 1 *161 (1947)*

19 Enders G and Hergft L Mineralzusammensetzung der Blutplattchen und weissen Blutkorperchen *Zeit f Biol* 88 *451 (1929)*

20 Fahraeus R Suspension stability of blood *Physiol Rev* 9 *241 (1929)*

21 Vejlens G Distribution of leucocytes in vascular system *Acta path et microbiol Scandin suppl* 33 *1 (1938)*

22 Levaditi C Sur l état de la cytose dans le plasma des animaux normaux et des organismes vaccines contre le vibrion cholerique *Ann Inst Pasteur* 15 *894 (1901)*

23 Aynaud M Action des microbes sur les globulins *Compt Rend de Biol* 70 *54 (1911)*

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2 BIZZOZERO J Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung *Virchows Arch f Path Anat 90 261 (1882)*

3 EBERTH J C and SCHIMMELBUSCH C Experimentelle Untersuchungen uber Thrombose *Virchows Arch f Path Anat* 103 39 *(1886)*

MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES OF PLATELETS AND HEMOSTASIS

MARJORIE B ZUCKER*

Department of Physiology New York University College of Dentistry

MY DISCUSSION WILL deal with the methods used to study platelet alterations and the results of these studies rather than with the contribution of platelets to coagulation

I would like first to emphasize the difficulty of obtaining platelets in an unaltered state — that is as they appear in the circulation Some of you I know are familiar with the work of Aynaud (1 2) He must have had superb technique because using paraffined equipment but no anticoagulants he was able to obtain platelet plasma from the donkey in which the platelets at 37 C appeared as batonnets At room temperature they appeared as discs I would like to ask Dr Ferguson and Dr Tocantins whether they understand better than I the distinction between batonnets" and discs As far as I can determine it depends upon whether the platelets are seen on edge or not

Ferguson These appearances were discussed in a paper of some years ago (3) Our feeling was that Aynaud s batonnets"† were platelets seen edgewise especially if they happened to have developed a short filiform excrescence at one or both ends

Zucker However I would not have thought that "batonnet" shaped platelets had excrescences any more than do platelets circulating in blood In any event Aynaud felt that temperature alone made a difference in the appearance of platelets

A second factor which according to Aynaud alters the appearance of platelets is the dilution of plasma If he diluted carefully

* Aided by a grant from the Life Insurance Medical Research Fund Some of this research was carried out in the Department of Physiology College of Physicians and Surgeons Columbia University and was aided by a grant from the Baruch Committee on Physical Medicine to Columbia University

† Hal Downey in his *Handbook of Hematology* mistakenly translates this as "little baton Actually the word refers to a child s home made toy simply fashioned by pointing one or both ends of a little stick used to play the game which the English call tip cat and the Boer children of South Africa kennetje The pointed end enables it to jump into the air when struck with a larger stick While in the air it may be hit to as great a distance as possible and the distance paced off to make a score in the game

24 GOVAERTS P Agglutination of bacteria to platelets and their electric changes *Compt Rend de Biol* 88 *993 (1923)*
25 ROSKAM J Fonction antizenique plasma et globulins (plaquettes) *Compt Rend de Biol* 85 *733 (1921)*
26 ——— *Physiologie normal et pathologique du globulins (plaquette de Bizzozero)* Paris Les Presses Universitaires de France 1927
27 COPLEY A L and HOULIHAN R B On the mechanism of platelet agglutination *Federation Proc 4 173 (1945)*
28 JAQUES L B *et al* Silicones and blood coagulation *Canad M A J* 55, *26 (1946)*
29 OSBORN S B and WRIGHT H P A method of estimating the time at which a counting rate changes using a Geiger Muller counter *Brit J Radiol* 22 *110 (1949)*
30 WRIGHT H P OSBORN S B and EDMONDS D G Rate of flow of venous blood in legs measured with radioactive sodium *Lancet* 2 *767 (1948)*
31 ——— Changes in the rate of flow of venous blood in the leg during pregnancy measured with radioactive sodium *Surg Gynec & Obstet* 90 *481 (1950)*
32 ——— Effect of postoperative bed rest and early ambulation on the rate of venous blood flow *Lancet* 1, *22 (1951)*
33 OCHSNER A DEBAKEY M and DECAMP P T Venous thrombosis *J A M A* 144 *831 (1950)*
34 SIMPSON K Shelter deaths from pulmonary embolism *Lancet* 2 *744 (1940)*
35 ROSKAM J and FONTAINE R *Thrombose Veineuse* Paris Masson 1950
36 OCHSNER A D DEBAKEY M and DECAMP P T Venous thrombosis Analysis of 580 cases *Surgery* 29 *24 (1951)*
37 VOORHIES A B and PULASKI E J The fibrinogen B test and intravascular thrombosis *J Lab & Clin Med* 34 *1352 (1949)*

the Dowex column with saline and running the blood in before the saline runs out

The question of whether thrombin can produce platelet alterations was mentioned earlier today The subject is not settled because both Zatti(5) and Fonio(6) who claimed that thrombin produces platelet changes simply added thrombin to platelet plasma The changes in platelets may have been produced by plasma components altered by thrombin such as Ac globulin or by impurities in the thrombin Zatti's work however is of interest since it indicates that calcium is not essential for these alterations they occurred when thrombin free of salts was added to citrated plasma The action of calcium on washed platelets has been studied by Milstone(7) He found that washed platelets suspended in buffered saline became swollen and less distinct in outline over a period of several hours These changes were little or not at all accelerated by the presence of calcium chloride

Agglutination is another change which platelets may undergo This has been studied by Copley and Houlihan(8 9) Using washed platelets they found that both plasma and serum were able to produce agglutination although serum was active in higher dilutions They also reported that agglutination of washed platelets occurred after adding purified globulin prothrombin thrombin placental tissue juice and plasma with a high concentration of heparin Agglutination was not produced by albumin fibrinogen or thromboplastin According to Aynaud(1) platelets suspended in plasma may be agglutinated by a great variety of substances It seems to me that agglutination of platelets *in vitro* is of such a nonspecific nature that its study is not likely to indicate why platelets agglutinate under physiological conditions for example at the site of vascular injury

A more specific change than simple agglutination is viscous metamorphosis — the fusion which agglutinated platelets undergo As Dr Tocantins stated earlier this fusion has been studied by Wright and Minot(10) and as far as I know by no one else They found that recalcification of a mixture of washed platelets and oxalated plasma resulted in metamorphosis Serum two to ten hours old was able to produce metamorphosis but thrombin and thromboplastin were not

Pinniger and Prunty(11) have shown that fibrinogen is not necessary for viscous metamorphosis since metamorphosis occurred in blood from a patient who was completely afibrinogenemic I have obtained similar results in dogs whose fibrinogen was reduced to

collected citrated platelet plasma with citrate saline the platelets altered from "batonnets to discs even when maintained at 37°C I was reminded of this dilution effect when Dr *Tocantins* was discussing the changes in coagulation time which occur when plasma is diluted

The consensus seems to be that anticoagulants *retard* changes in platelets but do not completely *prevent* them For example Tonio (4) has used magnesium sulphate plasma to study platelet alterations and has observed the formation of spreader" forms and bizarre excrescences The majority of workers feel that in citrated blood too the platelets slowly alter This is in contradistinction to fixed platelets which remain in the shape in which they are fixed

As a result of these difficulties I have been unable to obtain morphologically normal washed platelets even when careful silicone technique was used If someone has been able to do so I would be very glad to hear about it and talk to him

Flynn How were the platelets fixed?

Zucker If you take blood either from a vein with careful silicone technique or from a warm fingertip and immediately put it into 3 2 per cent citrate with 10 per cent formalin you fix the platelets as they are seen in the circulation You see very thin flat round discs they look like coins This is also the appearance of unfixed platelets if they are observed immediately at room temperature The first change in the appearance of a platelet is the development of excrescences at the edge of the disc As the changes proceed the platelet becomes rounded and shows more and more excrescences

To recapitulate then fresh platelets either fixed or at room temperature appear as discs without projections Gradually these platelets alter even in citrated plasma kept in siliconed glassware Washed platelets prepared from citrated plasma by differential centrifugation in siliconed glassware are rounded with excrescenses I have been unable to obtain washed platelets in the form in which they appear in the circulation Perhaps it is by definition impossible perhaps a platelet in saline cannot have the same shape as a platelet in plasma

Platelets have also been studied in blood decalcified with Dowex 50 In agreement with Dr Quick s finding with amberlite plasma the platelets are not clumped However they are rounded with excrescences even when air blood interfaces are avoided by filling

and that the fibrin attracts platelets which then initiate coagulation with conversion of prothrombin to thrombin On the other hand the thrombin may be present in high enough concentration to destroy prothrombin

Alexander How did you measure the prothrombin?

Zucker With the one stage Quick method

Alexander On whole plasma?

Zucker Yes, we added fibrinogen of course

Alexander Only fibrinogen added or other factors also?

Zucker The fibrinogen may have contained impurities The work was done before the discovery of Ac globulin

There is still another method of studying platelet function which I have employed namely observing the formation of the hemostatic platelet plug which forms after cutting a blood vessel(13) The data are summarized in Table XVI

TABLE XVI

Hemostasis After Nicking Mesenteric Veins In Normal and Treated Rats

Preparatory treatment	Total No of Experiments	Frequency of Results	
		Platelet plug present	Permanent cessation of bleeding
None	15	15	15
Thrombocytopenic purpura	5	0	0
Heparin 500u/kg	10	9	3
Heparin 2250u/kg	4	2	0
Dicumarol	5	5	5
Dicumarol and low vitamin K	3	3	0
Fibrinogenopenia	13	8	4

Reprinted from *Am J Physiol* **148** *275 (1947)*

Essentially the procedure was to nick one of the branches of the mesenteric veins Within thirty seconds a white thrombus began to form at the site of the nick and grew large enough to arrest

about 10 mg per cent by the slow intravenous injection of large amounts of thrombin

Still another method of studying platelet function is the ingenious method devised by Dr Helen Wright the measurement of their stickiness to glass This has already been discussed by Dr Wright I might add that Dr Moolten has separated two substances called thrombocytopen" and thrombocytosin from normal spleen and other tissues The former is found in enormously increased concentration in the spleens of patients with idiopathic thrombocytopenic purpura According to Moolten(12) these substances when added to blood can affect platelet adhesiveness thrombocytopen decreases and thrombocytosin increases adhesiveness He has not however given full data on this subject

Best Has anybody repeated that work?

Zucker I don t know

Tocantins I think Dr Karl Singer reported on that work recently in Washington

Alexander Only about thrombocytopen but he did not remark about its effect on platelet adhesiveness as I recall He could not confirm it

Zucker Another way of studying platelets is to avoid the problem of their isolation and instead to observe them in the circulation This of course is what has been done in Dr Best s laboratory The formation of white thrombi in glass shunts inserted into the circulation is depressed by heparin and dicumarol I have found that it is also depressed in rats with thrombocytopenic purpura produced by antiplatelet serum(13) and in dogs made fibrinogenopenic by the injection of thrombin(14) Unfortunately these dogs showed not only fibrinogenopenia but also a fall in prothrombin and platelets count so that they did not prove to be particularly desirable experimental objects They did fail to develop thrombi in glass shunts and they also had very long bleeding times Their prothrombin levels were about 15 per cent to 30 per cent of normal which alone would not have produced so great a hemorrhagic tendency On the other hand it does complicate things a great deal

Flynn Why does the prothrombin decrease?

Zucker I don t know It may be because fibrin is deposited along the walls of the blood vessels as shown by Jurgens and Studer(15)



FIGURE 47 The same vessels as in Figure 46 nine minutes after the vein was nicked Bleeding ceased in one and one-quarter minutes and was renewed three times The last recurrence took place five minutes after incision A platelet plug is seen lying on the vein and local constriction of both artery and vein is apparent



FIGURE 48 Section of a similar artery and vein exercised one-half hour after incision and stained with phosphotungstic acid hematoxylin

bleeding in two or three minutes The thrombus lay for the most part outside the blood vessel the portion within the lumen appeared *to be washed off Constriction of the nicked vessel occurred which* was mainly the result of direct mechanical stimulation of the vessel In addition vasoconstriction of the neighboring artery was produced as a result of the liberation of the potent vasoconstrictor substances which are found in platelets (Figures 46 and 47)

In most experiments although the platelet plug remained in place bleeding began again through the plug as far as one could deter*mine and lasted for a fraction of a minute This renewed bleeding* often occurred several times In the usual type of percutaneous puncture wound renewed bleeding may be prevented by coagulation of the blood filling the wound tract This is indicated by Howard Zucker s histological studies of human bleeding time tracts(16) The accumulation of extravasated blood was prevented in my experiments by a continuous saline drip on the mesentery and this may have contributed to the high incidence of renewed bleeding It is of interest that Dr Brinkhous found that bleeding often recurs in hemophilic dogs

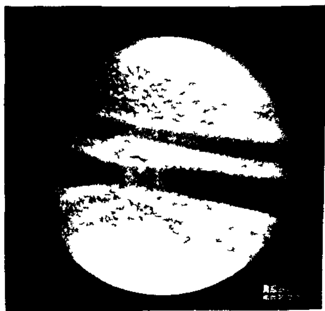


FIGURE 46* Mesenteric artery (above) and vein of normal rat One scale division equals 50 micra in this and subsequent figures

*Figures 46 47 and 49 reprinted by permission from *Am J Physiol* 148 275 (1947)

exsanguinated from the wound (Figure 49) In addition there was no vasoconstriction of the neighboring artery as there was in normal animals because of the absence of platelet vasoconstrictor substance

Similar experiments were performed on rats given 500 units per kg of heparin one half hour earlier In rats this dose prevented occlusion of an arteriovenous shunt but did not completely inhibit the formation of white thrombi on the shunt walls In most experiments platelet plugs formed at the site of vascular injury but in two thirds of these rats hemostasis was only temporary and the renewed bleeding continued indefinitely In one animal no hemostatic platelet plug formed

When 2250 units per kg of heparin were given a dosage which completely inhibited the formation of white thrombi on the walls of a glass shunt one half of the animals failed to form a platelet plug at all whereas the others formed plugs despite the high level of heparin However in none of these animals did effective hemostasis occur

When dicumarolized rats were studied with prothrombin times elevated from a normal of nineteen seconds to about thirty four seconds (one stage method prothrombin level 20 per cent of normal according to a saline dilution curve) hemostasis was entirely normal When however rats with prothrombin times of over three hundred seconds were used (diet low in vitamin K plus dicumarol) platelet plugs were observed but bleeding either continued despite the presence of the plug or was renewed after temporary cessation and continued indefinitely

Finally rats were used in which thrombin in very large doses was given intraperitoneally five hours earlier a method used by Smith and his co workers(17) The blood of these animals contained only traces of fibrinogen and in contrast to the dogs given thrombin the prothrombin activity was over 50 per cent of normal

In some of these animals hemostasis was normal In others no thrombus formed and bleeding did not cease In still others a platelet thrombus was present but hemostasis did not occur If one compares these animals with those given dicumarol it is evident that they are not bleeding because of prothrombin deficiency (insofar as the one stage method measures prothrombin) Whether they bleed because of the deficiency of fibrinogen or for some other reason I don t know I find these experiments very intriguing I do not know how to interpret them but I have a feeling that they

Figure 48 demonstrates a section of a nicked mesenteric rat vein and you can see that about half of the vein wall has been severely damaged The defect in the wall is covered by a large plug which is composed of fused platelets I draw your attention to the fact that in this plug there is no visible fibrin I cannot deny that there may be a film of unstainable fibrin but fibrin is not evident in this plug nor other slides of similar material In hemostatic plugs from the ear veins of dogs and in platelet plugs in humans(16) one does see bands of fibrin at some sites through the plugs

Kay Which stain is that? There are two different kinds of fibrin that may be stained

Zucker Phosphotungstic acid hematoxylin

I would like to question the prevalent notion that hemostasis is aided by syneresis of the intravascular clot In the first place the clot lies in the wall of the vessel and outside of the vessel but not to any great extent in the lumen Second there is so little fibrin in the hemostatic plug that I do not see how it could undergo retraction

When the veins of rats made thrombocytopenic by the injection of antiplatelet serum were nicked no trace of platelet plug was observed and no arrest of bleeding occurred The animals simply



Figure 49 Mesenteric vessels of purpuric rat (platelet count 12 000) one and one half minutes after the artery (below) was nicked Note the contraction of the artery the unchecked flow of blood and the absence of a platelet plug and of venous constriction Conditions remained unchanged until the animal died of blood loss twenty nine *minutes* after incision Nicked veins behaved similarly in purpuric rats In *normal* animals hemostasis in nicked arteries is identical with that in veins

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may contain important clues concerning the mechanism of hemo stasis

Finally I want briefly to mention a method which I am now using to investigate platelet function That is the study of the release of the vasoconstrictor substance(s) from the blood platelets It seemed to me that it would be very difficult to study the release of throm boplastin or Ac globulin from platelets since it is probable that substances involved in clotting will prove to cause their breakdown I thought it would be simpler to study instead the release of the vasoconstrictor substance

DISCUSSION

Laki Is there any clear cut proof that the vasoconstrictor sub stance is in the platelets?

Zucker There is a large literature on the subject One can obtain very potent vasoconstrictor extracts from platelets without clotting if you extract them with distilled water I would not call the sub stance a pressor substance since it does not seem to very effective in raising the blood pressure Its vasoconstrictor activity has been demonstrated on isolated vascular smooth muscle such as ox carotid artery and perfused cat tails and rabbit ears

Laki It is usually isolated from the serum

Zucker A substance has been isolated from beef serum by Rap port(18) probably 5 hydroxy tryptamine which is an exceedingly potent vasoconstrictor when measured on the perfused isolated rabbit s ear it is more potent than epinephrine under those circum stances He has not yet shown whether or not the same substance is in the platelets We are in the process of doing some work on this but from my own previous work and that of Reid and Bick(19) and others I think the evidence is very strong that the vasocon strictor substance is in the platelets and is released upon coagulation

Laki Do you introduce the platelet suspension into the blood vessels of an animal to test its effect?

Zucker I introduce an extract of blood platelets into the artery of an isolated perfused rabbit s ear This is such a sensitive indicator that one thousandth of a cubic centimeter of serum will cause a decrease in the outflow of the Ringer s solution perfusate

Laki Serum or platelet suspension?

Zucker I would not expect a suspension of intact platelets to be active However if one takes washed platelets adds them to plasma and recalcifies one can demonstrate release of constrictor substances

Laki It is still not clear to me You add the platelets to the plasma recalcify it and then note that after clotting there is some active substance This does not prove that it comes from the platelets

Zucker Not unless you do a control without the added platelets

Laki What happens if you just test the platelet suspension or extract alone?

Zucker Let me start this way One can demonstrate that there is much less vasoconstrictor substance in plasma than in serum However as Landis(20) has shown it is very difficult to obtain plasma completely free of any constrictor activity According to Reid serum prepared from plasma poor in platelets has far less constrictor activity than does serum prepared from platelet rich plasma Hence the substance appears to come from the platelets

Laki What bothers me is that I do not see a clear cut experiment to show that the platelets contain the active substance

Zucker If you separate the platelets from 10 ml of citrated blood (about 0 1 ml) extract them with distilled water and make the volume of the platelet extract up to the volume of serum which would be obtained from 10 ml of blood the platelet extract is about as potent as serum(21) Reid has done similar experiments

We have done a few experiments so far on the release of vasoconstrictor substance from washed platelets obtained from Dowex 50 blood The active material is not released by incubation of these platelets in saline containing 0 0025 M CaCl It appears to be released when the washed platelets are incubated with 10 units of Parke Davis commercial thrombin either with or without calcium We hope to go further with this work

Kay When you say Parke Davis commercial thrombin without calcium do you mean that you have actively removed the calcium? Parke Davis thrombin does contain calcium

Zucker That is a good point We did not remove the calcium from the thrombin I should have said that thrombin with or without *added* calcium does not liberate vasoconstrictor substance from washed platelets Do you know about how much calcium is present in the thrombin?

Kay No I don t I have never measured it but it is enough to precipitate an oxalate solution

I S Wright I want to tell you of an experience I had last night which might in a sense be a text for the Conference on Blood Clotting

Those who have the great advantage of being able to work without too much cerebral irritation in the laboratories sometimes miss the urgent stimulation presented when nature insists on being recognized

We have a patient with the rather unusual disease of *thrombo angitis obliterans* a female with a superimposed severe psychogenic overlay and the coldest leg and foot that I think I have ever encountered The foot constantly dripped sweat

We were unable to affect the combined spasm and organic occlusion by conservative measures We finally decided to perform a sympathectomy because by use of a sympathetic block it was possible to produce a very warm dry foot

A left lumbar sympathectomy was performed yesterday afternoon at one o clock My associates were there At seven o clock her right hand and arm went into complete spasm for inexplicable reasons except possibly that she had had an injection in a vein in her right forearm This hand became absolutely cadaveric in appearance and the arm was ice cold from elbow down

Many methods were used to attempt to break that spasm At ten the hand was still cold and presented exactly the problem this Conference is discussing Here we had the factors of a) possible irritation of a vein from the injection b) of a marked vasospasm possibly a reflex to conserve body heat and c) a severe psychogenic overlay with apprehensiveness on the part of the patient If this continued long enough we undoubtedly would have an irreversible change

Stellate blocks at first failed but later were successful when combined with dilaudide she was also given heparin and tromexan to prevent the static blood from thrombosing

Finally around two o clock the spasm relaxed and this morning I saw her and she had a very nice warm hand with a pulse down into the wrist

It is this type of problem that needles us to greater efforts in this field

If it had not been for the exploration of the laboratory workers the physiologists the anesthetists and the anticoagulationists this woman would probably have lost her hand

The need for combined knowledge and efforts of surgeons anesthetists and internists in order to combat such an acute emergency as this is apparent

This morning we are going to discuss antithrombin and alpha tocopherol and we have asked Walter Seegers to open the discussion

REFERENCES

1 AYNAUD M N *Le Globulin des Mammifères* Paris G Steinheil 1909

2 ——— Le globulin de l homme *Ann Instit Pasteur* 25 56 *(1911)*

3 FERGUSON J H Observations on alterations of blood platelets as factor in coagulation of blood *Am J Physiol* 108 670 *(1934)*

4 FONIO A *Die Thrombozyten des menschlichen Blutes* Bern Hans Huber 1942

5 ZATTI P Sulle cause dell agglutinazione delle piastrine nel sangue in via di coagulazione *Boll Soc ital biol sper* 24 22 *(1948)*

6 FONIO A Beobachtungen uber den Gerinnungs—und den Thrombosevorgang im Dunkelfeldnativpraparat *Schweiz med Wchnschr* 21 510 *(1940)*

7 MILSTONE J H Activation of prothrombin by platelets plus globulin *Proc Soc Exper Biol & Med* 68 225 *(1948)*

8 COPLEY A L and HOULIHAN R B The agglutination of platelets isolated from human dog and swine blood *Blood Spec Issue* 1 182 *(1947)*

9 ——— On the mechanism of platelet agglutination *Federation Proc* 4 173 *(1945)*

10 WRIGHT J H and MINOT G R The viscous metamorphosis of the blood platelets *J Exper Med* 26 395 *(1917)*

11 PINNINGER J L and PRUNTY F T G Some observations on the blood clotting mechanism The role of fibrinogen and platelets with reference to a case of congenital afibrinogenaemia *Brit J Exper Path* XXVII 200 *(1946)*

12 MOOLTEN S E VROMAN L and VROMAN G M S Adhesiveness of blood platelets in thromboembolism and hemorrhagic

disorders diagnostic and prognostic significance of platelet adhesiveness *Am J Clin Path* 19 814 (1949)

13 ZUCKER M B Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal thrombocytopenic heparinized and hypoprothrombinemic rats *Am J Physiol* 148 275 (1947)

14 ——— The effect of thrombin injections on hemostasis *Federation Proc* 7 138 (1948)

15 JURGENS R and STUDER A Zur Wirkung des Thrombins *Helv Physiol u Pharm Acta* 6 130 (1948)

16 ZUCKER H D Platelet thrombosis in human hemostasis A histologic study of skin wounds in normal and purpuric individuals *Blood* 4 631 (1949)

17 WARNER E D *et al* Further experience with the use of thrombin as a hemostatic agent *Proc Soc Exper Biol & Med* 41 655 (1939)

18 RAPPORT M M Serum vasoconstrictor (serotonin) Presence of creatinine in complex Proposed structure of vasoconstrictor principle *J Biol Chem* 180 961 (1949)

19 REID G and BICK M Pharmacologically active substances in serum *Australian J Exper Biol & M Sc* XX 33 (1942)

20 LANDIS E M WOOD J E JR and GUERRANT J L Effect of heparin on vasoconstrictor action of shed blood tested by perfusion of rabbit s ear *Am J Physiol* 139 26 (1943)

21 ZUCKER M B A study of the substances in blood serum and platelets which stimulate smooth muscle *Am J Physiol* 142 12 (1944)

ANTITHROMBIN — ALPHA TOCOPHEROL

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At the outset I wish to remark that the information about antithrombin which I have been able to obtain either from the literature or out of the laboratory has been especially difficult to acquire The work of Astrup Volkert and associates(1 2 3 4 5 6) was perhaps the most helpful because they dealt with fundamentals and gave impetus to the idea that more than one substance becomes involved in antithrombin studies There can be no doubt that one of the difficulties has been to obtain sufficient thrombin as a substrate with which to work But even after making it commercially available no one has taken the pains to work out fundamental relationships and the fundamental nature of antithrombin activity Instead there are a number of antithrombin tests that measure whatever is measured under the conditions of the experiment Simple tests are popular and often lead somewhere but the best simple test is most likely to be the one based on known fundamentals Who can now say what these various tests measure? What are the chances of having tests that measure with reasonable accuracy in both a qualitative and quantitative way? With the hope of furnishing a partial answer to these questions I propose to discuss several topics 1 What is thrombin? 2 The adsorption of thrombin on fibrin 3 The adsorption of thrombin on laboratory Glassware 4 The quantity of thrombin destroyed by plasma 5 Interference with the thrombin fibrinogen reaction by heparin and the plasma co factor 6 Antithrombin tests 7 Alpha tocopherol

I WHAT IS THROMBIN?

This question cannot be answered fully as yet However purification work and studies of the purified materials have been going on for many years(7 8 9 10 11) From this work we can get some idea of the probable nature of thrombin When purified prothrombin is activated autocatalytically by dissolving the product in a 25 per cent solution of sodium citrate one obtains at least three derivatives from the prothrombin when the electrophoresis technique is used as a criterion (Figure 50) One of these derivatives moves at

disorders diagnostic and prognostic significance of platelet adhesiveness *Am J Clin Path* 19 814 (1949)
13 ZUCKER M B Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal thrombocytopenic, heparinized and hypoprothrombinemic rats *Am J Physiol* 148 275 (1947)
14 ——— The effect of thrombin injections on hemostasis *Federation Proc* 7 138 (1948)
15 JURGENS R and STUDER A Zur Wirkung des Thrombins *Helv Physiol u Pharm Acta* 6 130 (1948)
16 ZUCKER H D Platelet thrombosis in human hemostasis A histologic study of skin wounds in normal and purpuric individuals *Blood* 4 631 (1949)
17 WARNER E D *et al* Further experience with the use of thrombin as a hemostatic agent *Proc Soc Exper Biol & Med* 41 655 (1939)
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20 LANDIS E M WOOD J E JR and GUERRANT J L Effect of heparin on vasoconstrictor action of shed blood tested by perfusion of rabbit s ear *Am J Physiol* 139 26 (1943)
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2 THE ADSORPTION OF THROMBIN ON FIBRIN

In some of our work on antithrombin the adsorption of thrombin on fibrin was not taken into account(12 13) and this led an an error in the constants of one of the equations representing the interaction of antithrombin and thrombin Studies on the adsorption of thrombin on fibrin were of importance from another point of view Howell(14) and Rettger(15) showed that there is a quantitative relationship between the concentration of thrombin and the amount of fibrin formed They did *not* discuss it in the light of an adsorption phenomena as recently said in the literature(16) They were thinking in terms of thrombin combining with fibrinogen as a part of the fundamental reaction Within limits the adsorption of thrombin on fibrinogen follows the equilibrium conditions found in adsorption reactions(17 18) Furthermore it is possible to get back practically all of the thrombin by lysing the fibrin clot with fibrinolysin for the latter apparently does not attack thrombin itself In any studies of antithrombin activity it is essential to take the adsorption phenomena into account but I do not believe that the disposal of thrombin by this mechanism is of much consequence either physiologically or when clotting goes at a normal rate in the test tube The main reason for this view is that the thrombin concentration never gets very high and adsorption equilibrium conditions are set up between a given low concentration of the thrombin in solution and the solid surface on which it becomes adsorbed Quite a number of years ago Dr Smith(19) showed that when prothrombin is consumed the thrombin concentration or thrombin tide as he called it reaches about 2 units per ml The equilibrium is therefore reached between the fibrin and a thrombin concentration of 2 units per ml It has been reasoned that thrombin is largely disposed by adsorption of thrombin on fibrin(16) and this was supposed to be good logic supported by the fact that Howell(14) prepared thrombin by taking the adsorbed thrombin off of fibrin clots That is most certainly true *but* how much thrombin did he obtain? Practically none by today s standards His work actually supports the view that very little thrombin is disposed of by adsorption The question then is where does the thrombin go? I think it is disposed of by antithrombin and not by adsorption on fibrin

3 THE ADSORPTION OF THROMBIN ON LABORATORY GLASSWARE

In connection with the discussion of this topic I am glad to see that Dr David Waugh is at the Conference because it was he who

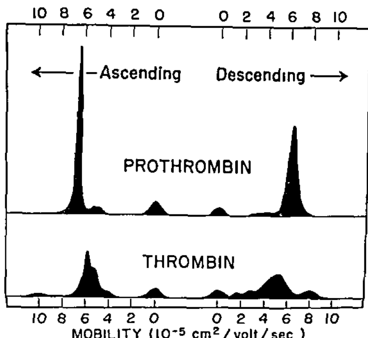


FIGURE 50 Comparison of electrophoretic patterns of purified prothrombin and of the same prothrombin after activation in 25 per cent sodium citrate solution

a very high rate in electrophoresis experiments It has an isoelectric point of 3 6 and we therefore refer to it as the 3 6 protein Electrophoretic separations of this component have been made on a microscale and this protein probably does not possess thrombin activity The other two components have their isoelectric points at pH 4 7 and 4 1 So far it has not been possible to separate them satisfactorily but the indications from electrophoretic separations are that both of these proteins are active About nine years ago(9) solubility studies performed with the best thrombin preparations then available also indicated that thrombin activity is possessed by two separate components Accordingly we are at present in the position of having inadequate evidence to support the view that two substances possess thrombin activity with no evidence to the contrary Any studies of antithrombin activity of plasma must take this possibility into account Also there exists the possibility that the 3 6 protein is important It can be seen in advance that any studies concerning antithrombin activity are temporarily on an uncertain foundation because there is as yet no adequate knowledge concerning the fundamental nature of thrombin itself and one can not describe the effects of something on a biological when that biological itself is not understood

the glass surface In the dilute solution the small quantities which become adsorbed are of significance relative to the original quantity there in the first place In the concentrated solution probably even more thrombin becomes adsorbed on the glass surface but this quantity is of relatively low significance with respect to the tremendous concentration of thrombin in the concentrate The amount adsorbed is so small that it is not noticed

Under the circumstances of the experiment recorded on Figure 51 it is only necessary to dilute the thrombin in a glass vessel lined with paraffin or some other substance that coats the surface

Laki Is it diluted with water?

Seegers It is diluted with nine tenths per cent sodium chloride but if you dilute it with water you obtain essentially the same type of answer

Then the diluted thrombin is stable If the dilution gives a 15 second clot in the beginning it will give a 15 second clot an hour later There is only one little detail which we do not understand The clot at the first minute is always appreciably shorter than the clot obtained from the sample two minutes later In describing these experiments the word adsorption has been used in a presumptive sort of way We have never been able to elute the adsorbed thrombin Actually all that is known from this experiment is that clean glass surfaces cause thrombin activity to disappear from dilute solutions Perhaps it is quite all right to continue referring to this phenomenon as adsorption on glass surfaces In many of our experiments on antithrombin and now also in experiments on prothrombin we take this possibility into account and take precautions against obtaining spurious results

Alexander May I ask what you use to coat your glassware Dr Seegers for standard procedures of assay?

Seegers We dont have a standard procedure of assay at the present time if you mean for antithrombin

Alexander No I am curious to know what coating you use for the practical assay for Ac globulin or prothrombin

Seegers By and large we have been using paraffin coated vessels but we are in the process of trying out all other kinds of other coatings that we do not have a true routine established on that We do not know what will eventually happen what we will eventually do in that connection

first suggested that thrombin is adsorbed on glassware Within the minute that this was mentioned it flashed through my mind that this phenomenon had been giving trouble for more than a decade What happens is illustrated by Figure 51 It is not possible to assay concentrated thrombin by mixing it directly with fibrinogen because all one observes is a rapid clot and it is not possible to make quantitative measurements The concentrated thrombin must first be diluted in such a manner that 15 second clotting will be obtained when the fibrinogen is added to the diluted thrombin Then by definition the concentration of the dilute solution is 1 unit per ml and it is possible to calculate back and assign a concentration value to the original solution It was always observed that the concentrated thrombin is stable whereas the diluted thrombin tends to lose its potency rather rapidly Within a few minutes a 15 9 second clotting time becomes 17 6 seconds then 18 2 seconds and so on A number of years ago it was simply assumed that the thrombin was not stable because it had not been freed of plasma antithrombin The more likely explanation is that the thrombin is being adsorbed on

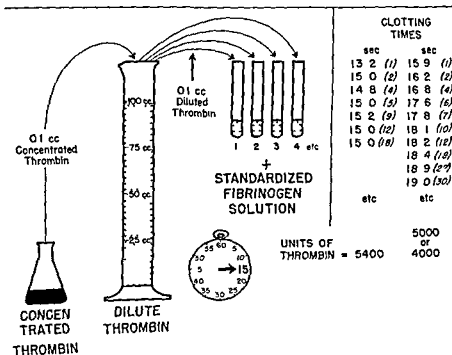


FIGURE 51 Arrangement for demonstrating the adsorption of thrombin from dilute solutions on laboratory glassware The data give a comparison of results with coated and noncoated glass containers

to a limited extent) with about 300 units of thrombin the thrombin activity disappears as shown on Figure 52 This is a situation where

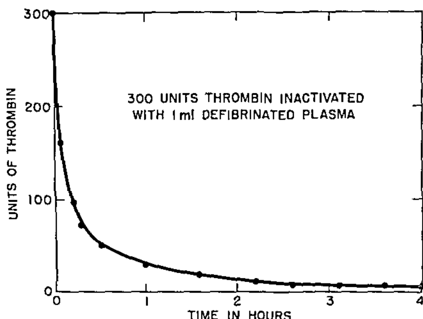


FIGURE 52* The inactivation of 300 units of thrombin with 1 cc of defibrinated bovine plasma Samples were taken at intervals to determine the total quantity of thrombin remaining

1 ml of defibrinated plasma is mixed with approximately that amount of thrombin which can be derived from the prothrombin ordinarily in that plasma Under these conditions the experiment is not influenced by adsorption of thrombin on fibrin Under these conditions practically all of the thrombin disappears within three hours Most of it is gone the first few minutes but even at the end of four hours some remains The last traces are disposed of with difficulty and theoretically a small amount of thrombin can exist free in plasma

If one mixes 1 ml of defibrinated plasma with 1750 units of thrombin instead of 300 it will destroy approximately 750 units of thrombin (Figure 53) The reaction takes place largely during the first fifteen minutes From this and other experiments it can be concluded that the quantity of thrombin which 1 ml of plasma can

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* Figures 52 and 53 reprinted by permission from *Blood* 5 [illegible]4 [illegible]5 (1950)

Edsall. Is silicone coating satisfactory?

Seegers. I don't know.

Waugh. It is satisfactory so far as we know.

Ferguson. We(20) have shown very definitely that progressive loss of potency in weak thrombin solutions is much lessened in siliconed vessels and markedly enhanced in the presence of powdered glass. We conclude that loss of thrombin due to adsorption on wettable surfaces must be considered together with antithrombin and thrombinolysis by potent proteases and micro-organism (e.g. mold) contaminants in reviewing factors which can operate antagonistically toward the stability of thrombin.

Laki. Would the disappearance of thrombin go on in the absence of oxygen?

Seegers. I haven't any idea. We have not tried any experiments to exclude oxygen. We do these at ordinary room temperature in the atmosphere in which we find ourselves in Detroit, which is about sea level. That is all we have done so far.

Tocantins. Are there any other substances like glass, silica containing substances such as Kaolin, that adsorb or inactivate thrombin?

Seegers. Well, I think I would prefer not to comment on that because we have a tremendous list of adsorbing agents that we know about and I cannot on the spur of the moment say which ones they are. We have information on perhaps forty or fifty of them and I could not carry that information in my mind.

Knisely. This may not be quite relevant but again and again this problem of adsorption on glass comes up. There is a large body of knowledge in the physical world on the surfaces of different kinds of glass. This is an extremely complex surface and it has complex charge effects. The men in the biological world who study the responses of lungs to quartz particles and other types of dusts know a great deal about those glass surfaces.

Seegers. I would then like to go on with the fourth topic.

4. THE QUANTITY OF THROMBIN DESTROYED BY PLASMA

If one mixes 1 ml. of plasma (that has been defibrinated with a small amount of thrombin or that has been defibrinated by heating

5 INTERFERENCE WITH THE THROMBIN FIBRINOGEN REACTION BY HEPARIN AND THE PLASMA CO FACTOR

In the two experiments cited above it was stated that heparin is of no influence in the reaction but it has always been said in the literature of recent years that heparin reacts with something in plasma to produce an antithrombin effect We believe that this mechanism involves interference with thrombin when it is reacting with fibrinogen and that thrombin is not destroyed by this mechanism Heparin and the plasma co factor presumably act together in a manner yet to be described in detail For example if one mixes 1 ml of plasma with 1750 units of thrombin the amount of thrombin which is destroyed is the same whether heparin is present or not When equilibrium conditions have been reached 1000 units of thrombin remain To assay for this 1000 units of thrombin the plasma must be diluted 1000 times and thereby the heparin in that same solution also becomes diluted 1000 times and is ineffective and does not show up in the thrombin fibrinogen reaction which must of necessity be used to measure the thrombin concentration If we go back to the first experiment (Figure 52) and mix 1 ml of plasma with 300 units of thrombin and also add heparin this heparin begins to have an effect on the thrombin fibrinogen reaction because the remaining thrombin plasma heparin mixture is not diluted very much before the fibrinogen is added Then we have interference with the thrombin fibrinogen reaction It is not necessary to review the literature which shows the effect of various concentrations of heparin on this phenomena Let me say that I do not believe that any information is available which may serve as a theoretical basis for the quantitative measurement of this co factor There is no quantitative literature about the interrelationships of heparin the co factor and interference with the thrombin fibrinogen reaction As a matter of fact some might question whether it is certain that this is the explanation of the co factor thrombin fibrinogen heparin situation We think that thrombin is not destroyed by this mechanism

It would of course be nice if these fundamental relationships could be worked out with a standard heparin preparation the purified co factor purified thrombin and purified fibrinogen It is my understanding that Dr Sylven and his associates may have the co factor in purified form and the outlook for the future would at the

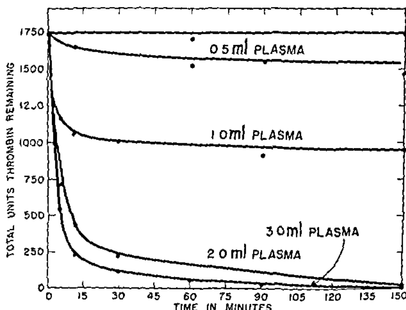


FIGURE 53 Reaction between various quantities of defibrinated plasma with 1 750 units of thrombin

destroy depends upon the quantity of thrombin which is there in the first place (21) The second fundamental conclusion which may be stated is that 1 ml of plasma is limited in its capacity to destroy thrombin even though any amount of time is allowed for that destruction(12)

It is of interest that Dr Kay and Dr Hutton state(22) that 0 25 mg of alpha tocopherol will neutralize about 3 units of thrombin In order to neutralize 750 units of thrombin plasma would then need to be a 6 per cent solution of alpha tocopherol

The addition of heparin does not alter the results The idea that plasma contains a substance which can inactivate thrombin and is independent of heparin action was clearly stated in the work of Astrup and Volkert cited above

No one has as yet described the fundamental relationship between thrombin concentration and antithrombin concentration for all combinations of the variables It should be possible to do this and it will be essential to do so before reliable quantitative work on antithrombin can be done

readily see that under the conditions described the antithrombin which acts independently of heparin can destroy thrombin then any heparin which might have entered the serum under physiological circumstances would exert its influence on the thrombin fibrinogen reaction and any heparin co factor which might be placed in or removed from the serum under physiological circumstances would also interfere with the thrombin fibrinogen reaction So it seems likely that there are at least three variables that can influence the results Volkert(6) used this method in their immunization experiments with rabbits in studies on the antithrombin content during and after anaphylactic shock in experiments on the influence of bile duct ligation in experiments on the influence of hemolyzed red cells on antithrombin activity and a variety of other interesting experiments They recognize the multiple nature of the factors influencing the test and indeed discuss some experiments as evidence of more than one antithrombin One cannot be certain which one of the substances in plasma contributed most to the figures obtained in any given experiment but a fairly good guess can be made For example in the anaphylactic shock experiment we probably see the influence of heparin in the early part of the experiment and the influence of the natural antithrombin ten to eighty hours after the shock dose was administered

By pointing out that the fundamental information which is required for quantitative antithrombin studies is not available as yet it is my hope that future authors will clearly state the limitations of their tests I do not wish to imply that empirical tests are worthless Certainly empirical tests can go a long distance and may be of considerable utility Let me recall the example learned in connection with prothrombin It was claimed that the one stage test measures prothrombin concentration quantitatively and among the arguments advanced in an attempt to prove the point it was said that its clinical usefulness had definitely proved its accuracy(23) Let us not again confuse utility and accuracy The tests that have been applied so far have been called antithrombin tests and I do not wish to propose that most of them might better be called the "Manhattan tests or any other name for differentiation or that they should be abolished The point that does need to be emphasized is that there is going to be any amount of difficulty misunderstanding and confusion if investigators who use empirical tests do not confine their remarks to the just limits of their data and those limits while wide enough for certain purposes are nevertheless limits that must be defined

moment seem rather bright from the standpoint of being able to obtain the required information

6 ANTITHROMBIN TESTS

There have been a large number of antithrombin tests devised and I do not believe that any of them are free of the following criticisms They measure multiple factors and are influenced to an unknown extent by one or more variables The variables I have in mind are a) the influence of the substance in plasma which acts independently of heparin b) the influence of heparin concentration c) the influence of co factor concentration and d) the possible influence of a large variety of unknown factors It would not be wise to review all of these tests Let me therefore take as an example the quantitative method devised by Astrup and his associates This method is not selected because it is a bad example but because it is probably the best method that has been devised to date They give careful consideration to the fundamental information available to them at that time and a good portion of that came from their own research work *In fact they advanced the concept of two main* antithrombin effects If I may be permitted a personal remark it seems to me a bit ironical that they should have reminded us that the concept of one antithrombin is not correct and while we were studying the possibility we discovered that their second antithrombin has all of the properties of fibrinogen Nevertheless our work still fits their conclusion which is that there are two main antithrombins

In their quantitative determination the following set up is arranged

TABLE XVII

Tube No	1	2	3	4
Thrombin*	1 00	1 00	1 00	1 00
Serum	0 05	0 10	0 15	0 20
0 9% NaCl	0 25	0 20	0 15	0 00

* About 40 units per ml

These mixtures are allowed to stand in a water bath at 37° for fifteen minutes Then samples are removed and mixed with fibrinogen to ascertain the quantity of thrombin remaining From the data they calculate the antithrombin concentration of the serum One can

tide the mixture could be injected and the clotting effect of thromboplastin was not observed Under certain conditions *in vivo* experiments were also successful in that large doses furnished protection against thromboplastin Fibrinogen counteracts the effects of each of these substances in test tube experiments

For the discussion I have some questions to ask and would like to place them in parallel with each fact

1 In those patients who show a low "antithrombin value thrombosis is likely My question is whether the alpha tocopherol concentration of their plasma is low or what substance is low in concentration?

2 The administration of alpha tocopherol phosphate with calcium gluconate increases the plasma antithrombin values I want to ask whether this increase is due to the administered alpha tocopherol which finds its way into the plasma and is then detected in the Kay test or whether the alpha tocopherol phosphate stimulates the physiological production of another antithrombin?

3 The administration of alpha tocopherol phosphate with calcium gluconate decreases the incidences of thrombosis Is that due to the physiological production of an unknown factor which is or may even not be detected by your antithrombin test?

In summary we do not know the exact nature of thrombin but most of the indications are that the activity resides in two separate proteins The substrate which is used in antithrombin studies is thus perhaps a mixture and one cannot be certain as to what this may mean in antithrombin studies The adsorption of thrombin on fibrin needs to be taken into account in antithrombin studies but for the reasons given this phenomenon is probably not of any consequence from a physiological viewpoint Under certain circumstances the adsorption of thrombin on glassware must be taken into account The word adsorption is used in the loose sense for all we actually know is that clean glass surfaces tend to make dilute thrombin solutions lose some of their activity The quantity of thrombin which is destroyed by plasma will vary with the quantity of thrombin available and heparin does not alter this quantity The antithrombic effect of heparin is probably manifested in conjunction with a co factor which seems to interfere with the interaction of thrombin and fibrinogen and this apparently does not involve destruction of thrombin So far no quantitative tests are available for measuring

It might not be out of place to raise the question of terminology

7 ALPHA TOCOPHEROL

My own experience with alpha tocopherol is quite limited and therefore I thought it might be in order to review very briefly the literature on the subject with the purpose of refreshing our back ground for the discussion later

Ferry and Shulman(24) have shown that a number of organic chemicals have a powerful inhibitory effect on the thrombin fibrinogen reaction Others have a potentiating effect Among these are gum acacia(25) phenol o cresol p cresol(26) Alpha tocopherol phosphate is one of these compounds which inhibits the action of thrombin(27) Zierter Grob and Lilienthal have shown that this occurs to a certain extent at a concentration in which the tocopherols are found *in normal human serum which is said to be about one* milligram per cent This antithrombic effect of alpha tocopherol could be demonstrated *in vitro* and *in vivo* in the latter case by the intraperitoneal injection of 50 milligrams per kilogram of body weight in rats With purified reagents Kay and Hutton(22) found that the inhibition of thrombin activity in the test tube can be reversed by the addition of calcium because calcium forms an in soluble compound with alpha tocopherol phosphate Kay and asso ciates(28) have devised an antithrombin test which is not based on fundamental relationships and is therefore an empirical test They present data to show that there is a thrombosing tendency when this test shows low antithrombin values Furthermore the administration of alpha tocopherol phosphate with calcium gluconate to patients causes the "antithrombin value to be increased The group of patients which received alpha tocopherol phosphate with calcium gluconate had a far lower incidence of thrombosis than a comparable group not so treated(29) They therefore advocate the use of this drug to deal with the problems presented by thrombo embolic phenomena It is not easy to decide whether the test tube experiments correlate with the clinical observations At first one is inclined to assume a direct connection but the evidence is incom plete

In another study(30) alpha tocopherol phosphate and inositol phosphatide were shown to have antithromboplastic activity The tests consisted of producing generalized thrombosis in mice upon injecting thromboplastin After thromboplastin was first incubated with monosodium alpha tocopherol phosphate or inositol phospha

tide the mixture could be injected and the clotting effect of thromboplastin was not observed Under certain conditions *in vivo* experiments were also successful in that large doses furnished protection against thromboplastin Fibrinogen counteracts the effects of each of these substances in test tube experiments

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the antithrombin concentration of plasma or the heparin antithrombin concentration of plasma accurately

DISCUSSION

Quick Mr Chairman may I make just one or two comments? First of all the adsorption of thrombin on glass I think is very important and I would recommend the following experiment for illustration Take ordinary thrombin put it in a glass test tube then pour it out and wash the test tube repeatedly with a saline solution

Take another test tube and add merely saline and then drain Put hemophilic blood into both of these test tubes It will be found that a hemophilic blood which clots say in fifty five minutes at 37° in the tube just washed with a saline will clot solidly in a time as short as eight minutes in the tube that had contained the thrombin Obviously repeated washings did not remove the thrombin which adheres to the glass walls and the amount is sufficient to initiate prompt clotting

The other comment that I want to make is that I think we have to be guarded when we say that the antithrombic action of fibrin is probably of no physiological significance That I think we will leave for the future to decide The scheme that I have presented I am not willing to disregard any more than in 1943 I was willing to discard the fact that another factor in addition to prothrombin reacts with thromboplastin and calcium to form thrombin At that time I unfortunately called the factor Component A which I later renamed labile factor

With regard to the one stage method it seems that no opportunity is missed to make a disparaging remark about it I still claim that the one stage method in almost all of the common conditions measures prothrombin quantitatively

I think I have published enough work on dicumarol vitamin K deficiency and so on to show that this test is a quantitative measure of prothrombin

I S Wright Dr E Warner

Warner First I would like to join Dr Seegers in a plea for careful quantitative work and insofar as possible with purified reagents in attempting to work out the mechanism of antithrombic action

The knowledge that we have of the mechanism so far has come mainly from successes along these lines First there was the dis

covery of heparin co factor with partially purified reagents Then some years ago working with a better thrombin we found that heparin did not increase the amount of thrombin destroyed by antithrombin with the particular reagents we used We did think that the heparin increased the speed with which thrombin was destroyed Working with different reagents and probably better reagents Astrup and his group have found that the increased or enhanced speed of the co factor action by heparin is not true with respect to the action of normal antithrombin

That observation recently has been corroborated by Dr Seegers s work with still other reagents and with probably the most highly purified thrombin that anyone has worked with

Dr Seegers stresses the point that in order to get a quantitative measure of the amount of thrombin destroyed or inactivated or removed we must deal with an excess of thrombin Since the action of thrombin on fibrinogen is the only end point we have in clotting studies we must have enough thrombin left to give an end point after the reaction is complete

Dr Seegers stresses very properly I think that there must be a thrombin excess in order to carry out quantitative studies in working out the mechanism Also dealing with purified reagents insofar as possible is important

In this connection I think it should be stressed that insofar as the clotting mechanism versus platelet agglutination possible sludge formation and so on enters into thromboembolic disease as we see it in man that we probably are dealing with the reverse situation That is we have probable traces of thrombin and an excess of anti thrombic substances under the conditions of clotting which takes part in thromboembolic disease For this reason relationships which can be worked out with high thrombin concentration may not hold up too well in the human case At least such relationships need to be questioned from the standpoint of the reversal of the quantitative relationship from excess thrombin on the one hand to traces of thrombin on the other

Further I suppose it should be pointed out that where sludge stagnation of blood and so on may be concerned in developing thrombi the concentration of clotting factors as they exist at the site of the developing thrombus may be quite different from that of the blood stream in general The sample we take out of the vein

might well not be representative of what is taking place behind a platelet thrombus or in a platelet thrombus or in a sedimented sludge

I would like to ask Dr Seegers if he or anyone else has done any quantitative studies on the recovery of thrombin once it has been inactivated by normal antithrombin I am thinking of the old idea of the acid alkali freeing of thrombin from the so called metathrombin Have any such quantitative recovery studies been done? For example where 1 ml of beef plasma has inactivated or destroyed some 750 units of thrombin how much of that 750 units can be recovered? Is the amount recoverable a trace or is it a significant amount?

Second, I would like to ask Dr Seegers about the time factor in connection with glass absorption of thrombin in his experiments The experiment shown was carried out to thirty minutes with a rather progressive decrease in thrombic activity in the solution I would like to ask Dr Seegers if the decrease in thrombin would continue had he carried the time out further or if the loss indicated in the slide is the total amount that that amount of glass surface would take out?

Then in connection with the work of Astrup and his group there are three things that bother me a bit about Astrup's co inhibitor not the normal antithrombin but the co inhibitor which acts in conjunction with heparin Astrup and Darling found this co-factor in the albumin fraction(31) although not in the same fraction exactly as that containing the normal antithrombin Also they found the co inhibitor to be present for a brief period after clotting It disappeared rapidly after clotting with thrombin and at the end of ten minutes I believe it had disappeared but it was not gone immediately after clotting had occurred Finally as to its heat stability Astrup indicated(31) that the co inhibitor, although very heat labile and quickly destroyed at 56° once combined with heparin to form the heparin inhibitor acquires heat stability — comparable to that of normal antithrombin and should withstand brief heating at 56° I find it difficult to reconcile these properties of Astrup's co-inhibitor with its being fibrinogen

We have no adequate data on this subject We do have a few preliminary and rather inconclusive results Taking that last point the heat stability it would seem that if the plasma is incubated with heparin prior to heating it then according to the work of Astrup

and Darling(31) such 56 plasma should have the normal anti thrombin plus the heparin co inhibitor Having been incubated with heparin it now would be heparin inhibitor and as such it should withstand the heat Therefore we should have the additive anti thrombic effect of the normal antithrombin plus the heparin in hibitor in such 56° plasma Our preliminary and rather inconclusive attempts at demonstrating this have failed to support the existence of this separate heparin co inhibitor

We have worked with commercial Parke Davis thrombin which Dr Seegers found to be rather unreliable in his work and I would like to ask Dr Seegers if he has tried this particular approach to the problem

Seegers I am afraid that I cannot add much except that there is a good possibility that the co inhibitor of Astrup and Volkert could be fibrinogen that it has many if not all of the properties of fibrinogen

Our way of getting at the idea that there are two separate anti thrombin effects were really promoted by the work of Astrup and Volkert cited above When we began inquiring as to why our views could not be correct in the light of what they had discovered we came to the conclusion that we would have a good working hypo thesis by considering that their co inhibitor is fibrinogen That would then leave Astrup and his co-workers with only one anti thrombic effect But there are two of them So if you take one of theirs away and you still have one remaining then the question is what are you going to name that? It seems to me quite evident that what is left is the one that has been talked about for many years It is not easy to decide from their experiments what their co inhibitor is

I had an opportunity to discuss this at length with Dr Astrup and Dr Volkert this summer and I do not want to quote their thinking but I believe they also would agree that the possibility is open that their co inhibitor could be fibrinogen

We devoted our attention to the normal antithrombin that is to say the antithrombin which is not affected by heparin and have done nothing with the other

Now I suppose we will get much help from the work of Sylven (32) and his associates if they have the heparin co factor in purified form so that we are inclined in our laboratory to continue to go

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We were working with the inactivating effects of formaldehyde on both fibrinogen and thrombin and found that formaldehyde will react very rapidly with fibrinogen and very slowly with dilute thrombin at 4 to 5 units per milliliter and formaldehyde concentrations of about four tenths of one per cent But strangely enough if one elevates the thrombin level to 75 units per milliliter under the same conditions then formaldehyde apparently inactivates thrombin This is a reversal of the usual findings

Tocantins There is apparently not much inactivation of the thrombin on the glassware since Dr Quick s experiment shows that that thrombin still is active

Waugh It is disappearance of thrombin Presumably it is adsorption which is not accompanied by denaturation

Tocantins And the other reason is that even though thrombin may be adsorbed on glassware the rate of blood coagulation is much faster on glass than it is on paraffin where the thrombin is apparently preserved

Waugh I think there is a reasonable explanation for that phenomenon too I would defer going into it until this afternoon

Seegers There is one question concerned with whether or not thrombin formation had been observed and whether or not one could get the thrombin back I have done no further experiments on that subject at all I would only like to make the observation that the metathrombin experiments recorded in the literature involved extremely minute amounts of thrombin and if they did reverse the reaction I don t know that that would tell us much in the way of quantities of thrombin

Warner That is the question I was wondering about — if you had tried it on a quantitative basis Those old experiments were done with mere traces of thrombin and I wondered if you had tried to see what part of the 750 units per milliliter you might get back Is it merely a trace or could you recover an appreciable quantity?

Seegers We have never tried to get any of them back We are I think only in the beginning stages of knowledge concerning the quantitative relationship here and I think we are lucky to be able to see how much is destroyed without knowing how much you can get back

ahead with the normal antithrombin and let the other go for a while I know it doesn t answer the question I think the question cannot *be answered at the present time*

The other question was whether or not the adsorption of thrombin on laboratory glassware — the dilute solution effect — will continue *beyond thirty minutes*

We have tried the experiment but you cannot come to any conclusion for this reason If you start with a dilute solution you are in the 15 *second clotting ranges and it rises to twenty and then you* get into a range which is quite inaccurate for measuring thrombin activity so that you come to a point where you don t know whether you are measuring the thrombin that is remaining or whether you are getting an artifact So *with the methods that we have at our* disposal we are unable to answer the question Perhaps Dr David Waugh with some of his refined methods can add to that I should also like to know whether it is all right for us to say that this is adsorption on glassware or whether we should use some other term?

Waugh I think we are reasonably safe in saying that it is adsorption

Warner Suppose you put in double the concentration Dr Seegers? That is rather than having only one unit to start with if you put in two units will the absorption be greater?

Seegers I haven t done that experiment

Alexander Can you saturate the glass?

Waugh I think you do saturate it in concentrated solutions

Ferry I would like to ask whether you could store the thrombin if you had some inert protein like serum albumin present to cover *the glass*

Waugh Yes I believe so

Ferry Another question If you have thrombin in the presence of glycerol will the glycerol prevent it from being *lost through* adsorption on the glass surface?

Seegers I haven t tried that experiment

Waugh May I point out one more thing at this time the inactivation of thrombin can present a complicated situation

I believe that the protein can also be recovered in the alpha globulin fraction by methanol precipitation — at least I have been told that it has been recovered I haven t had an opportunity to test it myself as yet

With your permission I will proceed to the clinical work on thrombosis without further reference to the *in vitro* work

As far as the antithrombin test is concerned I think that Dr Seegers suggestion that it should not be called antithrombin test is a good one

As a matter of fact we have begun to call it a prethrombotic index not only for the reason mentioned by Dr Seegers but also because we are no longer *sure* that it measures antithrombin in any form

In our original work we were supplied 65 unit ampoules which were carefully assayed Using this particular thrombin source we reported both a one and a two stage technique the two tests correlating well However when we began to use a commercial thrombin which is the only source available to most investigators we found that the one stage technique which we had reported in two articles was unsatisfactory since the one stage and two stage tests did not correlate low values being attained more frequently with the two stage technique Above 1 16 the correlation was very good but 1 8 s were rarely seen with the one stage technique while they continued to occur when the two stage technique was used The difference between the two tests is fairly simple In the one stage technique the thrombin is diluted and added to whole plasma and the clot read In the two stage technique the plasma is diluted and a constant amount of thrombin added and after incubation fibrinogen is added and the clot read The amount of thrombin used in the two tests was essentially equivalent and the fact that the two tests do not correlate in low levels immediately raises the question whether or not we are actually measuring an antithrombin For this reason I would like to propose the name prethrombotic index for the test which we have described The thrombin preparations of course contain various amounts of the factors necessary to convert prothrombin to thrombin since the technique of preparing thrombin consists of absorption of prothrombin from plasma and subsequent conversion by the addition of calcium thromboplastin and greater or lesser amounts of Ac globulin If the two tests correlate it would be reasonable to assume that the thrombin was the substance responsible for the clot production However since the two tests do not

Ferguson In our experiments on the classical so called meta thrombin question we have attempted to recover thrombic activity after it had been lost through the action of serum antithrombin Both the old Morawitz acid alkali method and the Minot chloroform method do result in some recovery of thrombic activity However these agents are destructive of thrombin and this largely defeats the purpose for which they are used

Most recently we have tried unsuccessfully to release thrombin from metathrombin by digestion with fibrinolysin

I S Wright Earlier we fell into a trap which we have been trying to avoid Our discussors began to give papers As Chairman I must accept the full responsibility for this I therefore ask you to bear in mind the fact that the greatest value of these conferences is based on the free exchange of ideas This is hampered if a few use up all of the time Please confine any one discussion to ten minutes or less

Kay I will omit discussion of the antithrombic effect of alpha tocopherol phosphate(22) because this activity has been previously reported (27) I must say however that we were unable to prolong the clotting time of dogs or rabbits by administration of alpha tocopherol phosphate intramuscularly or intravenously

In the dog and the rabbit alpha tocopherol phosphate is lethal at about 300 mg per kilo (intravenously administered) and at that level there is no effect on clotting time

There are three problems that I think Dr Seegers covered but didn t list in order a) whether or not the test we use measures an antithrombin b) whether or not the test we use is of any use in predicting thrombosis and c) whether or not tocopherol has any effect on either thrombosis or the test level

I want to thank Dr Seegers for being so kind in his comments about the work we have done The reports that we have put out are not complete in proving that tocopherol function is an antithrombin

We have been able to precipitate with calcium a protein from the ammonium sulfate precipitated alpha globulin fraction The protein when dissolved in dilute oxalate solutions and combined with fibrinogen and thrombin gives us a substance which behaves physically and chemically as fibrinogen B Furthermore this substance contains tocopherol as assayed by the Emmerie Engle technique and on incubation with thrombin inhibits it

In all these experiments relatively impure materials have been used except that the fibrinogen was dialyzed for much of the *in vitro* work but not for use in the prethrombotic index determination The fibrinogen incidentally is anticomplementary and I am not sure just exactly what effect defibrinating plasma might have on the anti thrombin test as we do it

Edsall What fibrinogen preparation did you use?

Kay Armour's bovine fibrinogen throughout You cannot use human fibrinogen (Cohn's Fraction 1 from human plasma) It has a variable amount of antithrombic activity of its own and the end points are not reproducible Now for the evaluation of our clinical data We have what I am sure you all consider a relatively small control series The reason for that we will come to in a moment Figure 54 shows 246 cases of which 150 had so called antithrombin

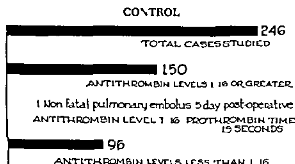


FIGURE 54

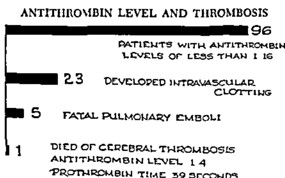


FIGURE 55

correlate the question arises whether or not in the two stage technique we are not measuring ability of lesser amounts of thromboplastin, calcium Ac globulin or thrombin to convert the prothrombin in diluted plasma At the present time I am unable to define the exact nature of the test which we use and therefore I feel it would be better to call it a prethrombotic index and to emphasize that if the test is to be used for prediction of a prethrombotic stage it must be done in two stages as follows

DETERMINATION OF PRETHROMBOTIC INDEX

1 Place in a test tube 1 4 ml of 1 85 per cent solution of $K_2C_2O_4$
2 Add 10 ml of blood as soon as it is withdrawn and mix well
3 Centrifuge at 1 500 r p m for ten minutes
4 Remove the supernatant plasma
5 Make a serial dilution of the plasma as follows
   a Place 0 5 ml of plasma in tube #1
   b Place 0 5 ml of plasma in tube #2 add 0 5 ml of distilled H_2O and mix well (1 1)
   c Place 0 5 ml of the mixture from tube #2 in tube #3 add 0 5 ml of distilled H_2O and mix well (1 4)
   d Continue to dilute the plasma in the manner described until ten tubes have been prepared The dilution in the tenth tube is 1 512
6 Add to each tube exactly 0 1 ml of thrombin solution This solution is prepared from Thrombin Topical (Parke Davis) The unitage on the label is a minimum assay and the exact unitage of ampoules can be obtained from Eugene Loomis of Parke Davis Research Laboratory Detroit Michigan If possible it is best to confirm the unitage by assay against bovine fibrinogen
7 Shake the tubes well then incubate them in a water bath at 37 5° C
8 Add to each tube 0 1 ml of 3 per cent bovine fibrinogen (Armour and Co) solution from which profibrin has been removed by freezing Shake well
9 Incubate the tubes for another sixty minutes
10 Observe each tube for clot formation recording the greatest dilution at which it ceases to occur

In normal plasma clot formation ceases to occur when the plasma dilution is between 1 32 and 1 128

First in the two stage technique the amount of the prothrombin did not correspond as you all know with the prothrombin conversion rate as measured by the one stage technique When we used the acetone extracted rabbit brain thromboplastin we found that almost all of our patients had normal or very near normal prothrombin times When we used the lung thromboplastin we found that only about 20 per cent of the patients whom we had at Charity had normal prothrombin times postoperatively At that time *we were using a lung thromboplastin* which gives us a 16 second normal If the prothrombin time was less than twenty seconds and the patient also had a prethrombotic index less than 1 16 about one half of those tested then showed some sign of intravascular clotting

More recently we have begun to use a different thromboplastin preparation On the basis of the work done by Dr Thomas(33) we hope to get a component by the use of oxalate and sedimentation We tried to get rid of accelerators by simply letting thromboplastin stand Various thromboplastins (some commercial and some laboratory prepared) were simply allowed to sit in the icebox with the hope that the accelerators in them would disappear As you can see from Table XIX they did not Actually over a period of

TABLE XIX

Effect of Time on Thromboplastic Activity
Prothombin Times of 1 Mouse Unit

Dates—1950—	7/15	7/21	7/28	8/4	8/18	9/8	9/12
Beef Lung	35	48	47	47	50	23	34
Beef Brain	75	77	52	55	31	44	89
Maltine	60	60	82	87	53	56	—
Schiefflin	30	28	21	25	17	35	45
Difco	36	30	22	33	20	25	52

* After heating 30 min at 56 C

time a mouse unit begins to show more thromboplastic activity as measured in terms of prothrombin time Now that does not indicate that the thromboplastin activity is not disappearing during that time In other words it took more of the original solution to kill the mouse or to produce a convulsion in the mouse than it did

levels of 1 16 or greater (I really believe that some term such as prethrombotic indices would be more satisfactory) and 96 had levels below 1 16 Figure 55 shows that of these 96 patients 23 developed some form of intravascular clotting Five of them had fatal pulmonary emboli and one died of cerebral thrombosis The incidence *as you will note is very high this we will explain later in* another figure There has been considerable difficulty in reproducing this test I am not quite sure why insofar as we are working with half dilutions and it takes almost 100 per cent error or it could take 100 per cent error before it would show up in the final reading However several investigators have been able to reproduce it

Table XVIII shows a series of cases from Dr Bregenser in Pittsburgh These are routine hospital admissions They have no significance other than to show that someone else has a significant series in which he was able to show that some patients had *1 8 and 1 4 levels*

TABLE XVIII

Antithrombin Levels

LEVEL	CASES	
1/4	3	1%
1/8	48	2 2%
1/16	1 529	70 6%
1/32	580	26 8%
1/64	6	3%

Total 2 166

Referring again to Figure 54, the 23 *patients who developed* intravascular clotting in addition to a low level necessarily fell into a group which had normal or near normal prothrombin times

We have had considerable difficulty in finding a satisfactory method for prothrombin determination Some lists which members of this Conference have suggested have been unsatisfactory in our hands It is not my plan to present a new prothrombin technique but merely to show why we have been unable to use some of the techniques that are used

and the supernatant fluid is then spun at 14 000 r p m for thirty minutes It is washed in distilled water and recentrifuged The oxalate removes the serum thromboplastin inhibitor Following this procedure the thromboplastin is resuspended in distilled water and heated at 56° for from thirty minutes to three hours The actual time makes very little difference in terms of the prothrombin time in relation to one mouse unit Heat stable accelerators and the inhibitor inositol phosphatide are not removed by this treatment You will see that for most tissues with the exception of beef lung the range of the prothrombin time of one mouse unit is relatively constant

Method B consisted of the same treatment of the tissue under study after acetone extraction Attempts to salt out a standard preparation with ammonium sulfate or sodium chloride were unsuccessful

The shortest and most satisfactory thromboplastin that we have found has been prepared from the cat lung We feel that prepara tion is free of the serum inhibitors except for inositol phosphatide In mice we can recover all the activity of a thromboplastin incu bated in serum once it has been oxalated

With this technique we have found that patients with pro thrombin times under thirty five seconds are apt to exhibit throm bosis when their antithrombin level gets below 1 16 One other thing I would say In our hands this thromboplastin has been useful since we are able to pick up patients who have prothrombin times with this cat lung preparation of twenty twenty two and twenty five seconds compared to a thirty five second normal These times have been found in patients with migratory thrombophlebitis and while they don t help in the diagnosis they suggest that there is some process involved that does shorten the prothrombin conver sion rate in that particular disease

The only other comment I would like to make at this time is that we have been unable to confirm Dr Jacox report using this thromboplastin If we add an optimum amount of this thrombo plastin to serum the clotting time of that mixture added to plasma is almost invariably longer than the prothrombin time done with the same thromboplastin We have seen two patients where this time was short One of these died of pulmonary emboli though I do not believe that this is of any significance

at the beginning of the experiment Then after heating the particular thromboplastin which had shown increasing activity in terms of prothrombin times of one mouse unit we were again able to prolong the time which indicates that either by bacterial contamination or by some not fully understood mechanism some accelerator which was heat labile had accumulated

For this reason we felt that we should make an active effort to eliminate the heat labile accelerators Various tissue components shown in Table XX were prepared as follows (Method A) The

TABLE XX

Prothrombin Time of 1 Mouse Unit

Methods	A	A+(NH) SO ppt	A+Nacl ppt	B
Beef Lung	35 (average) 73 79 70 75		* 180 + (50% Sat)	180+* 69
Beef Brain	77 87		* 180 + (50% Sat)	180+*
Cat Lung	30-35 34-36 33 36 35 39 33-38 35-41	70 78 (10 25% Sat) 80 86 (10% Sat)	* 180 + (50% Sat)	92 98 92 98* 36 48 81 84*
Cat Kidney				75 80
Dog Lung	52-64 60 64	110 120 (10-25% Sat) 120 130 (10% Sat)		135 145 18+*
Dog Kidney				100 105
Rabbit Lung	32 36 35 37			77 79 100 128
Rabbit Brain	40-47			42-47
Cat Lung Areas of Pneumonitis	49-50 55 60			

* Less than 1 mouse unit

material (lung brain kidney) is placed in 100 ml of water and 1 ml of 1 85 per cent of sodium oxalate solution is added This suspension is then spun at low speed of 2500 r p m for ten minutes

Table XXII shows the results of treatment One hundred and twenty two of these cases were run concomitantly with control cases However during that four month period we had four fatal emboli one in an eighteen year old girl She had a low antithrombin level and we all examined her and found no evidence of thrombosis but the next morning she was dead of pulmonary embolism At that time we wondered about the advisability of continuing a control group and I did a very unscientific thing I discontinued it However I believe I was justified

TABLE XXII

Results of Treatment

	Cases
Levels 1 16 or above	387
Levels below 1 16 (*Before treatment*)	3
Phlebothrombosis (*Presumptive*)	3
Emboli	2
(1—*Primary cause of death*)	
(1—*Incidental autopsy finding*)	
Total	395

I am not presenting this data as statistical proof that tocopherol is the answer to thrombosis We will have to study many more cases before I think we can consider it an improvement also I am anxious to have someone else take the material and use it as we used it and see if they can reproduce our results

The data are sufficient to indicate that thrombosis is apt to occur in one out of four or five cases when the prethrombotic index is below 1 16 and that it is very unlikely to occur when the index is 1 16 or above The data also indicate that routine administration of alpha tocopherol and calcium is sufficient to maintain the levels of most patients postoperatively The data indicate that we have influenced the incidence of thrombosis and embolism but the series is not of sufficient size to prove this point unequivocally

Dr Habif here in New York tells me he was unable to reproduce our results but I find that he has not used the dosage that we described

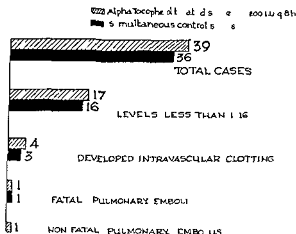


FIGURE 56 Reprinted by permission from Kay J H Balla G Hutton S B and Ochsner A *New Orleans M & S J* **103** *116 (1950)*

Figure 56 shows what happens to our levels when tocopherol is used alone We do not use the tocopherol phosphate in all patients We use it only when the patients cannot take oral medication The patients who can t take oral medication are placed on tocopherol intramuscularly administered You can see that when tocopherol is used alone there is essentially no effect on either the levels or the incidence of thrombosis

Table XXI defines the dosage of tocopherol and calcium which we use The alpha tocopherol phosphate must be given intra muscularly since intravenously it has no effect

TABLE XXI
Treatment

ALPHA TOCOPHEROL (*Epsilan M*)
200 I U q 8 hrs orally
OR
ALPHA TOCOPHEROL PHOSPHATE
(*Epsilan Phosphate*)
100 mg q 8 hrs I M
CALCIUM GLUCONATE
10cc of 10% q 24 hrs I V

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I S Wright For the record Dr Kay will you outline your dosage right now so that some of us who might wish to try this will be sure we are doing it exactly as you would do it?

Kay We put the patient immediately postoperatively on tocopherol every eight hours

I S Wright How much?

Kay If the patient can take oral medication we give them 200 i u every eight hours If the patient cannot take oral medication we give them 100 mg of the water soluble tocopherol intramuscularly The tocopherol in oil instead of the oral preparations has a very marked effect on antithrombin levels but it s the wrong way

I S Wright Will you tell me why that is? I was very much interested in your article which implied that the water soluble preparations worked in one direction and the oil preparations worked in the opposite direction

Kay We have evidence unpublished so far that if we add one half per cent phenol or chlorbutanol as preservatives to a multiple dose impoule we get the same lowering of the prethrombotic index with the water soluble preparation — in other words it suggests to me that there is a certain degree of toxicity in the preservatives that we use normally frequently in multiple dose ampoules

I S Wright In other words there was phenol or "globutnal in the preparations you have used

Kay Yes Now we have further experimental evidence that is statistically significant to show that these materials added to tocopherol will increase the mortality in experimental appendiceal peritonitis and we are getting that ready for publication now

I S Wright And how much calcium do you give?

Kay We have used the neocalglucon prepared by Sandoz I imagine that any preparation is satisfactory but I am telling you exactly what we use so that you will have the information

I S Wright Ten per cent?

Kay It s so-called ten per cent

I S Wright And how often do you give that and how?

Kay Intravenously every twenty four hours

I S Wright How much?

Kay Ten ml Now the reason why that is necessary is a little hard to understand and the fact that it works I think probably is about all I can say about it

I have a good many ideas and theories of why the calcium is necessary We know that when tocopherol is placed directly in the blood *in vitro* it has no effect on the antithrombin unless you get up into a terrifically high range of tocopherol However if you add a trace of the calcium salt of alpha tocopherol phosphate which is quite insoluble to plasma you then are able to measure by our technique an increase in the titer

That is the clue that started us using calcium intravenously and again all I can say is that it did work

Warner That is the calcium plus the tocopherol or calcium alone will increase the titer?

Kay Calcium plus tocopherol The calcium salt of tocopherol added to plasma will increase it However the increase is held pretty well down because of a relative insolubility of that tocopherol salt The highest levels that we can get are about 1 1024 that is one part of plasma in a thousand of water will inhibit 03 of a unit of thrombin

Overman I would like to ask one question What type of salt would you expect from alpha tocopherol and calcium?

Kay We dont know how tocopherol is carried in plasma

Edsall Is this tocopherol or tocopherol phosphate? The phos phate could form a salt

Kay Yes the alpha globulin — this is one of the loopholes and I know that you and Dr Seegers appreciate it This is one reason why we cannot carry the laboratory work over to the clinical work The work must be broken down into two separate studies The toco pherol in the alpha globulin fraction is assayable by the Emmerie Engle technique It is an alcohol there The phosphate which seems to inhibit thrombin is not assayable by this technique

Table XXIII shows the reason why our incidence is so high We have limited it to major surgical procedures The incidence prob ably is a little higher here than has been reported from other places

TABLE XXIII

Type of Procedure

	UNTREATED CONTROL CASES			TREATED CASES		
	Number	Thromboembolism Cases	Embolic Deaths	Number	Thromboembolism Cases	Embolic Deaths
Biliary Tract	51	5	1	88	1	
Alimentary Tract						
Esophageal Resection	3			2		
Gastric Resection	37	4		69		
Intestinal Resection	38	5		74	1	
Closure G I Perforation	9			35	2	1 contributory
Chest						
Pneumonectomy	22	3	2	22	1	1
Lobectomy	10			24		
Chest–Exploratory						
Non Malignant Diseases	12	1	1	11		
Maligant Diseases	6			12		
Abdomen–Exploratory						
Non Malignant Diseases	26			27		
Malignant Diseases	16	1	1	21		
Other	18	3*		10		
	246	23 (8 clots)	5	395	5 (no clots)	2

* Following radical neck disection endaneurysmorrhaphy and sympathectomy

but Dr Habif reports a 5 per cent incidence in this type of case and I don t believe we are too far out of line with that

You can see that we have reduced the incidence during this period to a point where I seriously wonder whether chance can still be a factor But I do not believe there is any denying that it can still be one

Knisely May I say something please? I deeply appreciate the way you have organized your data so that we can tell major surgery from minor surgery in evaluating the treatment

Kay That must be done The ultimate evaluation as we mentioned last night I think must be based on embolic deaths – autopsied embolic deaths The clinical diagnosis of thromboembolism is such a fleeting thing that there will never be any uniformity of opinion about it

Table XXIV shows that the inositol phosphatide which we have reported to be a good inhibitor of thromboplastin also raised the levels as measured by our prethrombotic index determination This is material from corn injected into dogs and mice There were very severe reactions to it and it was lethal at a given concentration

TABLE XXIV*

Antithrombic Effect of Inositol Phosphatide From Soya Bean

Dog #	Pre-Injection Level	POST INJECTION LEVEL			
		5 min	30 min	60 min	8 hours
1	1 64	Above 1 512	Above 1 512	1 256	1 128
2	1 16	1 512	1 256	1 192	1 128
3	1 16	1 256	1 128	1 128	1 64
4	1 32	1 384	1 384	1 192	1 128
5	1 64	1 256	1 256	1 256	1 128
6	1 32	1 256	1 256	1 128	1 64
7	1 32	1 384	1 256	1 256	1 128
8	1 64	1 512	1 384	1 256	1 256
9	1 16	1 256	1 128	1 128	1 64
10	1 16	1 384	1 256	1 192	1 192

* Tables XXIV and XXV reprinted from *Bull Tulane Med Faculty* **10** *76 (1951)*

However recently we have obtained an inositol phosphatide from soy beans which is not toxic in any dosage we have used as yet

Table XXV shows that a single intravenous injection will maintain all our levels at much higher values than we have been able to attain with anything else for periods up to eight hours

TABLE XXV

Antithrombic Effect of Inositol Phosphatide From Corn

Dog #	Preinjection Level	POST INJECTION LEVEL			
		5 min	30 min	60 min	8 hours
1	1 32	1 128	1 64	1 32	1 32
2	1 16	1 64	1 16	1 8	1 32
3*	1 64	1 128	1 64	1 64	1 64
4	1 16	1 32	1 16	1 16	1 16
5†	1 32				
6	1 16	1 128	1 64	1 32	1 16
7	1 64	1 128	1 64	1 64	1 32
8	1 32	1 64	1 32	1 16	1 16
9,	1 16				
10	1 16	1 64	1 32	1 16	1 16

* Dog recovered following reaction
† Dogs died following reactions

Tocantins I should like to point out that the soy bean phosphatide that Dr Macfarlane worked with did not have antithrombin activity(34)

Kay This is an antithrombic effect of inositol phosphatide as measured by our test which we don t feel actually measures antithrombin

I see that that is going to be very confusing but that is the situation

I S Wright Are there any questions?

Seegers In those patients who show a low amount of antithrombin my question is Is it the tocopherol concentration of a plasma that is low or is it some other substance that is low in concentration so that the tocopherol concentration need not be low?

Kay As to what substance is low in concentration I can only say that I don t know

Seegers The administration of alpha tocopherol phosphate increases the plasma antithrombin level I wish to ask whether the increase is due to the administration of alpha tocopherol which finds its way in plasma and is then measured in the test or whether it stimulates the physiological production of another antithrombin

Kay I can only say that our data do not answer the question It seems unlikely that it stimulates another antithrombin — no I can t say that The fact that the preservative does away with its effect might give some clue but it needn t The preservative could interfere with the stimulation of another substance equally as well

Seegers The administration of alpha tocopherol phosphate with calcium gluconate decreases the incidence of thrombosis Is that due to the physiological production of an unknown factor which may or may even not be measured by your antithrombin test?

Kay This last is harsh I feel that the test does measure a factor which permits us to predict with some accuracy those patients who are most likely to develop thrombosis However I do not have statistical data to prove that we have decreased the incidence of thrombosis

Alexander May I ask Dr Kay whether you can correlate the progressive decrease in antithrombic" titer with progressive dates postoperative?

Kay No

Barker How often were the tests done?

Kay The tests were done every day of the patient s hospital stay following surgery The antithrombin level goes down only about one day Sometimes we will see it down for two days running These patients develop thrombosis within a short time after that phenomenon

Barker Do you mean on the same day or on the next day?

Kay On two occasions I have had the opportunity to obtain the low level One was a ward patient who was complaining of pain in the calf when taken to surgery a clot was found at operation Another case was a patient whom we had looked over very carefully in the afternoon for evidence of thrombosis the next morning or during the night she had a fatal embolism

I S Wright For how long do you test for antithrombin activity?

Kay As long as the patient is in the hospital

I S Wright You mean three weeks?

Kay We don t hospitalize our patients that long as a rule Following a gastrectomy they usually go home in seven days

Warner In those who have developed the low level postoperatively how close to the time of operation has that been immediately postoperative or has it been usually some days later?

Kay It has been any day as a matter of fact, anywhere from the first day following surgery When a patient stays three weeks it may be the twenty first day

Warner Has there been evidence of thromboembolism soon after this change?

Kay We have seen clinical signs develop within any time from the period we get the level up to seventy two hours later

Warner So that your clinical evidence of thromboembolism has been pretty well distributed generally from the time of operation on?

Kay Yes

Warner And one other question In the patients who developed the low level have you had any that you did not treat and if so is the low level transient?

Kay It is transient It comes back of its own accord and once we get a low level it is very often too late to begin therapy

Alexander The possibility is still open that the change you observed in titer may be a consequence of a clot having formed?

Kay No we have never seen a low level in a patient who had clinical evidence of a clot In other words in order to get the low level you must run the test before the clot has formed The observation is of no help in diagnosis It s a good prognostic test but no help in diagnosis

Alexander I gather that the development of a low titer is almost coincident in time (within twenty four hours) with the demonstration of a clot?

Kay It can be but not always Clinical signs sometimes occur as long as seventy two hours after

Alexander If not always is it not quite possible that the clot has failed to be detected clinically?

Kay Well that is always a possibility However to illustrate in the morning we go around draw the blood ask the patient how he is and ask about and examine his legs After determining the level we go back and examine the patient If the clot was there that morning clinically I was unable to demonstrate

Alexander We all know it may be there and yet there may be no clinical signs

Kay When we get clinical manifestations of thrombosis the level is always normal

Best I find this extremely interesting and one can understand that clinical groups when they have a new substance or a new procedure wish to establish it or throw it out as quickly as possible But I cannot quite see why instead of untreated controls you can not use controls treated by the best known treatment I mean take half your cases and say give them heparin and dicumarol and the other half the new treatment that you are working on

Kay Of course some of you are familiar with the argument about ligation versus anticoagulant therapy It so happened when I first went to work on this problem we were using ligation as a method of therapy so that these controls were all ligated when they developed clinical signs of thrombosis Despite that fact we had five emboli which I think is an indication that ligation alone isn t adequate On the other hand had we used heparin once a clot developed or once we had clinical signs of the disease I do not think we would have done any better because again these patients who had emboli had not shown clinically anything that would make us suspect that they needed any anticoagulant

If you are talking about the routine treatment of any postopera tive patient then I would say that that would be all right

Best That is what I have been talking about

Kay That would be a control group but it would not be a control on the incidence of thrombosis

Best I mean that you might take your major operations and divide them into two groups To one you give whatever you like —

heparin or dicumarol and on the other you try the new treatment You may require more figures to get an answer but you probably would not have to blame yourself if a fatality occurred

Kay But I don t see that that would be a control group It would be a control on the effectiveness of heparin or dicumarol which has already been studied

Best Well it would be a comparison of the two methods of treatment

Kay But not a comparison of the incidence of thrombosis

Best I don t know that we want to know that We want to know the efficacy of a new procedure

I S Wright As compared with the best that previously existed

Best Yes

I S Wright That seems fair to me

Best If you were going to try a new treatment of diabetic coma — would you do a control group and let them all die or would you do the best thing you know and compare the new procedure with that?

Kay I don t feel too strongly about it but I do not feel we can use dicumarol on our wards

Best Well you apparently felt strongly when you had an un treated case which died

Kay That isn t what I am starting to say I say that I don t feel strongly about the use of anticoagulants

However many members of our department at Tulane do not feel that the clinical use particularly in Charity where we have poor control in terms of prothrombin times that the routine clinical use of dicumarol would be advisable

Best I am only speaking because you threw away your scientific method at a certain point in your study I thought perhaps you could use controls of another type i e treated by the best known methods rather than not treated at all

Kay I see your point and I leave it to the Conference whether control with a substance such as dicumarol and heparin would add anything other than a comparison between two techniques which it was not our desire to make

I S Wright Dr Best has raised a very important point In order for this therapy to be accepted it must be proven to be better than previous therapy — not just better than no treatment

I am aware that you are associated with a group who have publicly on numerous occasions expressed an antipathy or fear of dicumarol therapy

Those familiar with the use of dicumarol and heparin use them constantly and feel that these fears are exaggerated if the team work and laboratory conditions are satisfactory

However I think Dr Best s point is very well taken Ultimately you are going to have to make the decision as to whether you are going to compare it with the best that we have previously

It was like the dicumarol test for coronary thrombosis We didn t deprive the patients of oxygen of transfusion if they needed it of stimulation and let them die — we gave them the best treatment that existed prior to that time and then half of them received in addition what was thought to be the thing we were testing We didn t know whether it was going to be any good or not but that was our test Dr Overman you have been working with alpha tocopherol for some time Would you give us some of your results?

Overman We have been determining vitamin E levels in the blood after the oral and intramuscular administration of several derivatives of dl alpha tocopherol *† It has been possible to increase the level of tocopherol from approximately 1 mg per cent which is the normal level to over 3 mg per cent by the oral administration of dl alpha tocopherol acetate The intramuscular injection of dl alpha tocopherol phosphate mono sodium salt has not increased the free tocopherol level in the plasma

This is probably due to the fact that the phosphate derivative is very stable and probably resists hydrolysis in the body When Karrer(35) originally prepared dl alpha tocopherol phosphate in 1940 he indicated that it was very difficult to hydrolyze The intramuscular injection of *dl alpha tocopherol in sesame oil does not* give an increase in plasma level of tocopherol either

* From the Department of Medicine Cornell University Medical College aided by grants from the Kress Hampil Hyde and Lasker Foundations and Hoffmann La Roche Inc

† We wish to acknowledge the valuable assistance of J M M Neely M E Todd and W H Roeder in this investigation

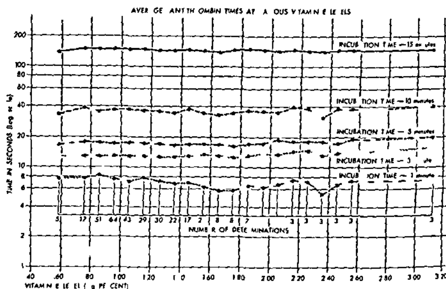


FIGURE 57

The antithrombin titers were also determined by three methods — Quick, A. J. (36), Astrup, T. (4) and Kay, J. H. (37) — on these plasmas at the various levels of dl alpha tocopherol as indicated in Figure 57. The antithrombin times presented in the Figure are values obtained by the method described by Quick.

The thrombin solution used contained approximately 20 units per milliliter. This concentration was used because it gave an antithrombin time for the fifteen minute incubation period of around one hundred and forty seconds. It was thought that any slight changes that might occur in antithrombin titer should be more easily detected at this range. The number of determinations at the various levels of tocopherol are also indicated on Figure 57. No change in antithrombin titer was detected in the plasmas with a tocopherol level of 0 60 mg per cent to 3 2 mg per cent at any of the five different incubation periods. The same results were obtained using Astrup's and Kay's method.

At present we are studying antithrombin titer of two groups of patients who have undergone surgery. One group is being treated according to the method used by Ochsner (tocopherol and calcium) and the other is the control group. Vitamin E, calcium and antithrombin titers are determined daily on both groups. So far we have not detected any change in antithrombin titer in these two groups.

A review of the evidence thus far presented and the experience of critical investigators fails to demonstrate that alpha tocopherol derivatives act as anticoagulant substances when administered to man or have any effect on the prevention or treatment of thromboembolic conditions

Kay Are you using the one stage technique or the two stage?

Overman We have been using the exact method that you published in the *New Orleans Medical and Surgical Journal*

Kay That is one of our mistakes I ask your forgiveness for that That is the one stage technique and at that time with a specially prepared 65 unit ampoule it gave us identical results with those of the two stage technique

However this fall when we no longer had this specially prepared thrombin we found that the two tests no longer did correlate and we had to use the two stage technique

Seegers I wonder whether Dr Kay has ever put some animals on a vitamin E deficiency diet to see what would happen? It seems to me that in a study of this kind it is very essential that laboratory work be done and one of the logical things to do would be to see what happens to the blood of vitamin E deficient animals

Kay I haven t done it There aren t many animals large enough to permit daily withdrawal of a few milliliters of blood over a long period time which do not synthesize their own vitamin E

Olwin I would like to say first in passing that we have never found any correlation between a high prothrombin and a tendency of the patient to develop intravascular clotting Assays have been done for the most part with the two stage method In our experience women during pregnancy may develop an elevated prothrombin to as high as 178 per cent and not show evidence of intravascular clotting It seems to me that there is a lot of feeling over the country that that may be an important factor and I should like to say that in our experience there is no evidence for such a contention

Kay I wish to state that with the two-stage technique we couldn t demonstrate anything significant It s only when we used a relatively slow one stage prothrombin time that this correlation existed and as pointed out it was relative and not absolute

Olwin Now on the subject of antithrombin we as surgeons are interested in preventing intravascular clotting of course and when any new method of treatment or prevention is presented we feel that we should look into it We have been interested in methods and the validity of methods and so when Dr Kay s reports appeared or even before some of them appeared we wanted to do studies similar to his

We studied the blood of patients undergoing major surgery We used pre and postoperatively in parallel the technique of Dr Kay and that developed by Dr Seegers and his associates for the assay of antithrombin Dr Kay s method is the one described about six months ago(29) and involves the dilution of thrombin rather than the dilution of plasma For purposes of discussion I shall refer to this as the one stage method Dr Seegers technique is based on the second phase of the two stage prothrombin assay and I shall refer to it as the two stage method We are reluctant to call it an antithrombin assay but it involves the assay of thrombin prior to incubation with various dilutions of plasma usually 100 per cent 75 per cent 50 per cent and 25 per cent and then the reassay of those mixtures following incubation for twenty minutes at 29° The difference between the thrombin unitage before and after incubation is recorded as the amount destroyed by that specific plasma *or dilution of plasma and this point is plotted on a graph* Individual points for the various dilutions of any single plasma are plotted as a curve or as it develops a more or less straight line Using the two techniques we have studied cases usually preoperatively the first day postoperatively and alternate days thereafter for varying periods depending on the length of stay in the hospital

We have deviated from Dr Kay s technique somewhat for the following reason He tells me that he is at present using a thrombin specially prepared by Parke Davis and Company Until recently however the material used was the commercially available thrombin Ampoules of this material bearing the same lot number when assayed by the Iowa method(38) may vary in unitage in our laboratory from 2000 to 5500 per ampoule So that if you dilute an ampoule of this thrombin and I am sure a number of workers who are using this test are using commercial thrombin — if you dilute it on the basis of the unitage given for that lot you don t know what strength of thrombin you are using Hence we have assayed the thrombin just prior to its use and have found that a

concentration of 1 unit per ml was not sufficient to give us a clotting in some of the tubes and no clotting in some of the other tubes Two unit per ml thrombin however or shall I say thrombin that varies from 1 5 to 2 5 units per ml will give us that so called normal clotting pattern That is in two tubes clotting is present and in three tubes there is no clotting

We have also used thrombin that has been diluted with a solution containing 50 per cent glycerol and 50 per cent salt solution the reason for that being that such thrombin is relatively stable when stored at 4°C It gives results similar to those obtained with thrombin diluted with distilled water or salt solution Thrombin diluted with either of the latter is relatively unstable

First I should like to say that we have up to the present time found no uniform variation in the so called antithrombin in our postoperative cases In some instances patients have shown a lowered antithrombin and in some cases the antithrombin has increased postoperatively We have had no uniform variation in the incidence of intravascular clotting in those cases but our series is much too small to be of any significance Figure 58 represents the antithrombin variations in a single surgical patient before and in the first week following major surgery The figures on the left represent the tech

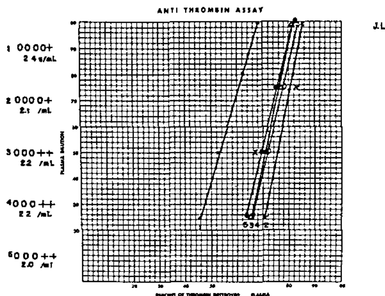


Figure 58

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to be fairly resistant to dilution As you see here at 25 per cent there is a 45 per cent destruction and at 100 per cent there is a 65 per cent destruction of thrombin

Line 1 Figure 58 represents a low so called antithrombin Line 2 indicates the first postoperative level an increase in the so called antithrombin and then the plasma reverts to slightly less anti thrombic activity in subsequent determinations as shown by lines 3 4 and 5

Now as compared to this Dr Kays technique gives the following results Lines 1 and 2 of the two stage technique indicate a low and high antithrombin respectively The results of the one stage technique are the same for both plasmas — indicating a high antithrombin Lines 3 4 and 5 are in about the same bracket and the corresponding samples according to the one stage technique each show clotting in the two right tubes

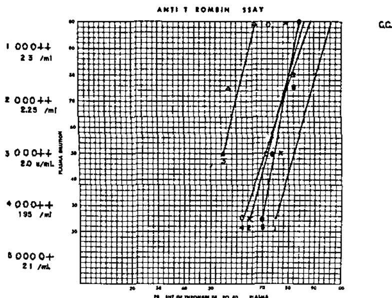


FIGURE 59

Figure 59 shows another patient and the lines here represent similar days following surgery line 1 being the preoperative level line 2 the first day postoperatively line 3 the third postoperative day et cetera In this case according to the two stage technique you can see that the antithrombin is highest preoperatively (line 1) and lowest on the third day following surgery (line 3) The remain

nique of Dr Kay the technique that he was using something over a year ago In each instance there is represented under the results the unitage per milliliter of thrombin used in that particular assay

The points on the graph represent the results of the two stage technique The ordinate represents the dilution of the plasma in per cent of normal and the abscissa the thrombin destroyed by the individual plasma full strength or diluted The individual lines represent the results obtained with a single blood sample Line 1 indicates the pattern for the preoperative assay line 2 the first assay postoperatively either the day of surgery or the day following and lines 3 4 and 5 represent the assays on the second fourth and sixth days following the first postoperative assay Sunday may move one or two lines back one day as we do no assays on that day

Flynn Does the thrombin unitage shown on the chart represent the amount you started with or the amount in the final clotting tube?

Olwin The abscissa represents the per cent of thrombin destroyed

Flynn I mean in the ordinate there?

Olwin The ordinate represents the concentration of plasma The top line is undiluted plasma that is 100 per cent plasma one quarter of the way down is 75 per cent plasma half way down is 50 per cent plasma that is plasma diluted once with saline Each of these different plasma dilutions is incubated with a thrombin solution having about 750 units per milliliter The thrombin solution is assayed just prior to and just following incubation

Alexander In diluted plasma? Seven hundred units of thrombin added per milliliter of plasma or diluted plasma?

Olwin Thats right Is that clear?

Flynn Yes

Olwin This follows in the main the technique that was described by Dr Smith and Dr Seegers some years ago and more recently by Klein and Seegers(38) and I should say that normally whole plasma will destroy about 70 to 90 per cent of the thrombin with which it is incubated When various plasma dilutions are used the lines in general slope gently towards the left as the dilution of the plasma increases The so called antithrombic factor normally seems

Alexander Have you found any change in the ability of the plasma to handle additional thrombin in relation to the postoperative period?

Olwin Not uniformly

I S Wright Dr Barker do you have any comments on antithrombin or tocopherol?

Barker A few years ago Margaret Hurn Frank D Mann and I(39) did some work with a test for so called antithrombin using a slight modification of the method of Astrup and Darling Single tests were done in 47 supposedly normal individuals and 57 patients who had evidence of very recent arterial or venous thrombosis In the latter group some values were obtained which were higher and some which were lower than any in the control group but the mean of all values was essentially the same in the group with thrombosis as in the group of normal individuals

I would like to comment briefly on methods for evaluation of therapeutic procedures for the prevention of postoperative thrombosis I agree with Dr Kay that comparative incidences of thrombosis and embolism diagnosed by clinical methods in treated and untreated groups of patients is subject to considerable error The comparative incidence of fatal pulmonary embolism is subject to much less error if a large percentage of careful necropsies are done However the incidence of fatal postoperative pulmonary embolism in untreated patients is small Even after such extensive abdominal operations as gastric resection and colon resection our incidence has only been 0 7 per cent or seven in one thousand and after cholecystectomy it has been 0 3 per cent or three in one thousand Thus if the incidence of fatal pulmonary embolism is used to evaluate a procedure used for the purpose of preventing thrombosis a very large series of patients must be treated before one can conclude that the procedure is or is not of value Some years ago we used thyroid extract during the immediate postoperative period since it increases the circulation rate in the lower extremities as measured by determinations of circulation time No fatal pulmonary embolisms developed in the first 1 000 patients who had had cholecystectomy and were given thyroid extract but in the next 100 patients so treated three died of pulmonary embolism which brought the incidence up almost to that of the control series

I S Wright We ran into similar studies conducted with anticoagulant treatment of coronary occlusion where the death rate we will say is approximately 25 per cent

ing assays fall between these two extremes On the other hand the one stage technique shows no variation in antithrombin from the preoperative level through the fifth postoperative day On the seventh day there is an increase in antithrombin as you see in the lower left hand corner

These are fairly representative of the findings up to the present time We still are not willing to place any definite interpretation on them but the study will be continued and we hope eventually to have an answer to the question in the first place, does either of these tests give us information as to whether or not they will help us anticipate clotting postoperatively and second will they be equally valid if such be the case?

Kay I think when you are using the two stage test that we use you will be able to reproduce our results Do you know for sure—did you use fibrinogen in this test?

Olwin You mean in your technique?

Kay Yes

Olwin No this is the one that you described about fifteen months ago perhaps and was published last year

Kay Not the first one that was described In July of this past year the one stage technique was published for the first time You will just have to forgive me because as long as we used that one type of thrombin the two tests correlated perfectly I must say that we tried the glycerol saline suspension of thrombin in the original two stage test and we could not get reproducible results with it We never obtained low levels unless we used a water solution of thrombin

Alexander Dr Olwin I should like to ask whether I infer correctly from your data that in the first day postoperative the plasma has a greater ability to inactivate added thrombin in the amount of 700 units of thrombin per ml than preoperatively?

Olwin We dont say that We have no uniform results that will allow us to make any such conclusions at this time

These data are representative of the respective results between the two tests These are not representative of what actually happens to the patients so called antithrombin

nature labels them in a manner that is comparable to radioactive element tagging of molecules Consequently when any function of blood clotting is related to immunology and its serum proteins my interest increases tenfold

Dr Kay in your description of reagents and purification techniques you made a remark to the effect that your thrombin had an anticomplementary effect

Kay Serum thromboplastin inhibitor behaves as a complement

Haberman Why did you make that remark?

Kay I think what I had in mind concerned a point made by Dr Knisely that we would have to break down the sludge phenomenon and decide whether it was a factor of blood coagulation or a factor in the immune reaction that was responsible for it I don t believe that it is necessary to make that differentiation or to put it another way I think it is conceivable that they are much the same

For instance the serum thromboplastin inhibitor behaves in many ways as a complement It has a marked effect on surface tension * It can be increased in serum by the addition of a small amount of calcium and has an optimum calcium concentration(40) In the presence of oxalate there is no thromboplastin inhibitor in the presence of fibrinogen the serum thromboplastin inhibitor is lost and in the presence of fibrinogen there is no lysis of cells

Edsall That means that if you use plasma instead of serum you cannot carry out tests for complement activity?

Kay That is true I believe Dr Haberman knows more about that

Haberman I wouldn t say that — In some studies that we have made on banked blood it was found that one can get into difficulty with complement titrations on plasma My beliefs come from these studies which indicate that it s not the plasma that interferes with complement but that it is the anticoagulant that is used Almost everyone of these that I have tested was strongly anticomplementary in the concentrations used If you desire to use plasma in complement titrations one way to do so in the face of anticoagulants is to use large amounts of complement in order to saturate the anticomplementary activity Even then it isn t too satisfactory

Edsall You can decalcify your plasma with an ion exchange resin

* Unpublished data

In cases treated by the best previous methods from individual hospitals we received figures of 35 or 50 patients where the death rate might range anywhere from 5 per cent to 60 per cent It wasn t until the total reached 250 – then 400 500 and 600 cases that the curve began to level off and from then to 1000 the over all figures were almost identical They varied very little I want to emphasize that many series of coronary occlusion have been re ported of 100 or fewer cases some treated with anticoagulants and some without attempting to prove or disprove some point We can take small blocks of patients from certain hospitals and prove almost anything But when the figures for 600 patients didn t vary from those for 700 800 900 or 1 000 the statisticians pronounced the results valid

That was with a group of patients where the mortality rate is 25 per cent which would give you a more valid statistical analysis than where your anticipated incidence is only 2 or 3 per cent Under those conditions it becomes very difficult to establish sta tistically valid figures unless you study many thousands of cases As Dr Barker pointed out a shift of only one or two affect your percentage enormously and make valid conclusions difficult or impossible

Kay That s very true I don t want to suggest that our statistics are final in any way I would say though that as far as hospital admission incidence goes we apparently are luckier than you are because only 0 0176 per cent of our patients die of embolism

I S Wright That is over all admission?

Kay Yes over all admission Now however when you begin to consider the number involved during the year 1949 we had on an average slightly more than one death per month on our surgical service at Charity That number of patients begins to look like an awful lot of people dying when you see them every month We have been a lot luckier this past year – whether it s been weather phenomenon or due to tocopherol I can t say

I S Wright Any further questions or comments on this point Dr Haberman?

Haberman I would like to leave the clinic and return to the laboratory My main interest in blood clotting is that this mechanism is largely composed of what might be called tagged" protein mole cules The activities of these large proteins along with their specific

tination" and "pseudoagglutination" and "capillary attraction and surface tension and of surface tension as a factor in a phagocytosis

Now I hope that that number of words is sufficient to stimulate Dr Edsall and Dr Waugh to present a brief set of ideas about these phenomena particularly so that they can append the bibliography The bibliography should lead us toward understanding "London forces" secondary valence forces and other forces which operate on molecules large molecules and small structures like platelets larger structures like cell surfaces — all of these phenomena which are being discussed this morning

Dr Warner talked about the possibility that the exact forces operating to cement things together in say "sedimented sludge" may be quite different from the substances and forces we find in samples drawn from static systems and I hope that this bibliography will give us some information which can be used in thinking about that set of problems

Edsall I m sure that other people will be able to think of references in addition to those I can suggest

Van der Waals forces and London forces are really the same thing London s contribution was essentially to develop a quantum mechanical theory which explained the nature of Van der Waals forces on a more fundamental level In any case such forces certainly operate between all atoms and molecules Even the inert gases attract one another by such interactions and so do all other atoms and molecules too These forces fall off very rapidly as the molecules get farther apart roughly with the inverse seventh power of the distance

Then of course if molecules are electrically asymmetrical or charged you get electrostatic forces of various kinds The interionic forces of course are the most far reaching since they vary as the inverse square of the distance and therefore act over relatively great distances

Then there are dipoles and dipolar ions such as the amino acids and proteins at their isoelectric points and smaller dipoles such as ordinary polar molecules like water nitrobenzene the alcohols the ketones and such things

In addition to these forces there are the forces that lead directly to the formation of chemical bonds These can be adequately defined only in the abstract language of quantum mechanics For

Haberman Yes that can be done I meant in the presence of such things as the citrates oxalates and ACD type solutions I have never felt that the anticomplementary effect was due to the fibrinogen

Kay We started with a 1 per cent fibrinogen solution and dialyzed it to get rid of citrate and then froze it to remove profibrin Now what the actual concentration of the fibrinogen was I dont know and in addition we dont know what the original fraction was The original fraction is not 100 per cent fibrinogen However let me put it this way This substance did interfere with the lysis of red cells in the hemolytic reaction

Haberman There is one other point that might be made on the use of such substances as anticoagulants and their effect on complement An anticomplementary effect may be observed in serum collected from an animal or patient in deep anesthesia without obvious changes in the clotting mechanism Also barbiturates can give an anticomplementary effect

I S Wright Dr Knisely wished to discuss the question of additional bibliography

Knisely My purpose in introducing the concept of whether this was blood clotting chemistry making red cells stick together or protein or rather specific immune proteins making them stick together was in order to get ideas on both groups

If you show a tree to a lumberman he talks about boards If you showed it to a botanist he talks about *xylem* and *phloem* If you show that tree to a chemist he may talk about cellulose or wood alcohol So let us get each one to talk in all directions to see what we can learn

Yesterday Dr Wright talked about problems of platelets sticking together and Dr Zucker talked about problems of platelets sticking to vessel walls This morning Dr Seegers talked about one of the blood constituents — thrombin — sticking to glass

This leads to assembling a basic bibliography of some literature from the world of physics and chemistry which is being used in biology but which is not sufficiently known to biologists

In biology we talk about adhesion" or cohesion about adsorption or "absorption and conglutination and we speak of agglu

tination and "pseudoagglutination and capillary attraction and surface tension and of surface tension as a factor in a phagocytosis

Now I hope that that number of words is sufficient to stimulate Dr Edsall and Dr Waugh to present a brief set of ideas about these phenomena particularly so that they can append the bibliography The bibliography should lead us toward understanding "London forces secondary valence forces and other forces which operate on molecules large molecules and small structures like platelets larger structures like cell surfaces — all of these phenomena which are being discussed this morning

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most of us there is certainly no need to delve into these matters we can get along with a simple working picture which is quite nonmathematical However there are important reactions involving for instance the combination of proteins with many smaller mole cules and ions which you can describe quite well in terms of the law of mass action Other interactions like the solvent action of dilute salt solutions on globulins can be described in terms of laws that depend on the ionic strength of the solution or on some func tion of the number and valence and size and charge distribution of the ions present in the system and also on the dielectric constant of the medium

Even if you can describe the combination in terms of the law of mass action it may still be just a kind of coupling due to electro-static attraction without the formation of a true covalent bond You can often tell the difference by spectroscopic studies since absorption bands are generally displaced when a true covalent bond is formed

As to books that discuss this field I think you do not particu larly want the more abstract and highly mathematical treatises Linus Paulings *Nature of the Chemical Bond*(41) Another valu able book that came out very recently in a new edition is Hilde brand and Scotts *Solubility of Nonelectrolytes*(42) Although the title speaks of solubility the book is largely taken up with the discussion of the nature of intermolecular forces in general with solubility as one particular manifestation of them

For dipolar ions perhaps the book by E J Cohn and me on proteins amino acids and peptides(43) which covers a rather large amount of material might be helpful

Water and its properties are so important biologically that I think I should mention the paper by J D Bernal and R H Fowler on the structure of water and of ionic solutions(44) Some of this is highly technical and some of the conclusions of the authors have had to be modified since but this paper is still a landmark in its field If you read it in conjunction with L J Hendersons *The Fit ness of the Environment*(45) you will get a pretty comprehensive picture of the biological importance of water in relation to its structure

You cannot discuss the structure of water, of course without dis cussing the *hydrogen bond* a subject of major importance in all

kinds of biochemical systems Pauling's book which I have already mentioned has an excellent chapter on the hydrogen bond(41)

Haberman What about long range forces?

Edsall Such forces do play an important part in some systems for instance in the parallel orientation of tobacco mosaic virus particles which can occur even in dilute solution when the particles are a long way apart(46) The theory is extremely complicated and even some very expert people have gone off the track in their mathematical calculations The best theoretical treatment is probably in a recent paper by Lars Onsager(47) I may say that for most people including myself it is an extremely difficult paper to read

Knisely Does that perhaps parallel the work of De Jong of Holland some time ago on co acervates?

Edsall I am no expert on the subject of coacervation I think Dr Ferry would be more competent than I to discuss it The phenomena are certainly important There is a good review on the subject by Deuschman(48) which is the most lucid discussion of coacervation that I have seen

Knisely Edsall one more thing Would you give us a few references in the higher mathematical range? Even though we cannot handle them we can see that graduate students read them

Edsall There are several quite good books intended to introduce nonphysicists to quantum mechanics The one I have used most is by Linus Pauling and E B Wilson Jr (49) I think it is a very good book You have to know the fundamentals of differential and integral calculus before you start to read it but you do not need more preparation than that

There is another book by V Rojansky (50) which I think gives a more elementary account of the philosophy of the subject as well as the essential mathematical development Henry Eyring John Walter and G E Kimball have written a book on the subject which some of my friends recommend highly(51) I have not used it myself

Link I would like to add as an agriculturalist – you see I am in a situation here comparable to the medics – that it has to be very simple in order for me to be attracted to it and simpler still for me to understand it

There is a book by Sexton(52) which has an interesting chapter on big molecules

Edsall I have seen some very favorable reviews of the book

Fink It is very good so it ought to be used Someone wrote me a while back for a list of books on enzymes that would be useful to an anatomist and I said that I did not know anything that happened in the field that was published after Auldenes book which came out in 1826

Knisely May we continue this one more moment please Dr Waugh has been thinking along this line and he had one or two books that have not been put in here and one or two attacks on the problem including Einsteins equation

Waugh I have very little to add to what Dr Edsall has said I think that we are facing a difficult problem One can treat the interactions of molecules at large distances rather well but then when the particles approach each other and the distances become small then the problems of interaction are quite different and one starts thinking in terms of specific surface configurations Of course the best example of this is the interaction of antigens and antibodies where if the two molecules are separated by 50 A their forces of interaction are quite different than if they are separated by 5 or 10 A

Incidentally I dont know of any good reference to the latter classification I think that Slater in his *Introduction to Chemical Physics* considers the secondary valence types of interaction

Edsall That is a very fine book

Link I might suggest to the people interested in the platelets and so forth that the book on plant physiology by Styles is very good on this general question of transmission of things through membranes — I mean it is good from the standpoint of the amateur

Knisely I would like to add one so called fact here — the observation is that in some kinds of sludge the mutual attraction of particles is so great that masses which have diameters on the order of magnitude of 50 micra will attract each other powerfully into groups from distances beginning as far as 50 to 1 200 micra apart in stationary blood This has been observed in living human subjects who had no operation and no touching of the observed tissue and no anesthesia There can be real forces here — the above are the largest observed but there are many many smaller ones

Flynn Dr Seegers there is one thing that bothers me about your discussion where you stated that Astrup s co inhibitor is fibrinogen It is my impression that Astrup and Darling showed fibrinogen free preparations to have co inhibitor activity

When they added such a preparation to heparin antithrombic effect was potentiated whereas in the control with heparin alone the inactivation was much less How can that be correlated with the viewpoint that there really is not such a thing as co inhibitor except fibrinogen? Do you know the work I refer to?

Seegers Well I have gone over all these papers by Astrup and Volkert and I am not sure that I know which one you are referring to

It is by no means a clear situation I exchanged views with the originators of that work and it doesn t clarify itself You do find in their work the statement that if they defibrinate plasma the co inhibitor is no longer there I don t know what that means I haven t any idea how it is going to come out I must say that I am inclined to believe we have good evidence that their co inhibitor is fibrinogen Many of the properties they describe are the properties of fibrinogen I think however that we are in a very poor position to say anything more about it because we have concentrated our efforts in the direction of the natural antithrombin and have dropped all of the other works for the simple reason that one can only do so much

Edsall Two or three years ago Dr Fritz Koller from Switzerland worked in our laboratory for a while and carried out studies which involved adding various plasma fractions with heparin to the clotting mixture of fibrinogen and thrombin The fraction called IV 1 which is largely alphaglobulin and contains an alphalipoprotein was found to have little or no antithrombic action without heparin But with heparin it did show a marked inhibition of clotting and subfractionation seemed to indicate that the activity went into a fraction that had the alphalipoprotein in it

I am not at all sure that this component is alphalipoprotein but Koller s work suggested that there is something in this fraction which contains no fibrinogen that does apparently serve to act with heparin to inhibit clotting

Seegers I hope that we have made it clear that we are not saying that this is not a substance in plasma that reacts with heparin We

certainly do think so As a matter of fact I suspected that it is the same substance that Dr Sylvén has in a concentrate and perhaps even in a purified form That is of course just guessing

Edsall Is Sylvén's work published yet?

Flynn It will be published in *Biochimica et Biophysica Acta** (53)

Ferguson I do think that Dr Seegers is rendering us a real service in clearly differentiating between the several types of mechanism which fall under the general scope of "antithrombic" factors The separation between those which involve heparin and those which do not is particularly important Since 1937 we(54) have stressed that the true antithrombic action of heparin plus its co factor is immediate in contrast to the progressive" inactivation by serum antithrombin (? albumin) and (experimentally) tryptic thrombinolysis(55 56) I believe our "immediate" antithrombic action is what Seegers refers to as interference with the thrombin fibrinogen reaction My erstwhile colleague Dr A J Glazko(56) sought to explain this inhibition upon ionic effects analogous to potassium ferrocyanide as discussed at an early Macy Conference(57) When comparing the more rapid loss of potency of thrombin added to heparinized serum as compared with simple serum we would stress that as the active thrombin is lessened through reaction (? combination) with serum antithrombin the immediate neutralizing effects of the heparin mechanism become more and more marked

Salmine (protamine) which can neutralize the inhibitory effects of heparin on the thrombin fibrinogen reaction is fibrinoplastic (58) but I should be hesitant to inflict the term "antifibrinoplastic" upon the mechanism of the immediate antithrombic action of heparin

Tocantins Do you think that the increase in platelets that takes place after surgical operations may lead to the presence in the serum of an excess of the platelet factor which Dr Seegers showed enhances the conversion of fibrinogen to fibrin by thrombin and thereby give rise to what may appear to be a decrease in the antithrombin activity of the serum?

Seegers It is a very complicated situation and I don t think we want to get into it now

I S Wright Dr Kolomon Laki will discuss fibrinogen and fibrin

* This paper has now appeared — *Editor*

REFERENCES

1 ASTRUP T Quelques observations sur la coagulation du fibrinogene *Bull Soc chim biol* 29 *391 (1947)*

2 ASTRUP T and DARLING S Antithrombin und Heparin *Naturwissenschaften* 29 *300 (1941)*

3 ASTRUP T SELSO S and VOLKERT M Bemerkungen uber die Bestimmung des Antithrombingehalts im Blute *Biochem Ztschr* 315 *303 (1943)*

4 ASTRUP T and DARLING S Measurement and properties of antithrombin *Acta physiol Scandinav 4 293 (1942)*

5 ASTRUP T and DARLING S Antithrombin and heparin *Acta physiol Scandinav* 5 *13 (1943)*

6 VOLKERT M Studies on the antithrombin content of the blood and its relation to heparin *Acta physiol Scandinav* 5 *Suppl 15 (1942)*

7 SEEGERS W H *et al* The purification of thrombin *J Biol Chem* 126 *91 (1938)*

8 SEEGERS W H Purification of prothrombin and thrombin chemical properties of purified preparations *J Biol Chem* 136 *103 (1940)*

9 SEEGERS W H and MCGINTY D A Further purification of thrombin probable purity of products *J Biol Chem* 146 *511 (1942)*

10 WARE A G and SEEGERS W H Studies on prothrombin purification inactivation with thrombin and activation with thromboplastin and calcium *J Biol Chem* 174 *565 (1948)*

11 SEEGERS W H MCCLAUGHRY R I and FAHEY J L Some properties of purified prothrombin and its activation with sodium citrate *Blood* 5 *421 (1950)*

12 SEEGERS W H *et al* Heparin and antithrombic activity of plasma *Science* 96 *300 (1942)*

13 SEEGERS W H and SMITH H P Antithrombic activity of plasma quantitative interrelationships *Proc Soc Exper Biol & Med* 52 *159 (1943)*

14 HOWELL W H The preparation and properties of thrombin together with observations on antithrombin and prothrombin *Am J Physiol* 26 *453 (1910)*

15 RETTGER L J The coagulation of blood *Am J Physiol* 24 *406 (1909)*

16 QUICK A J and FAVRE GILLY J Fibrin factor influencing consumption of prothrombin in coagulation *Am J Physiol* 158 *387 (1949)*

17 SEEGERS W H NIEFT M and LOOMIS E C Note on the adsorption of thrombin on fibrin *Science* 101 *520 (1945)*

18 SEEGERS W H Multiple protein interactions as exhibited by the blood clotting mechanism *J Phys and Coll Chem* 51 198 (1947)

19 SMITH H P The coagulation of blood *Essays in Biology* Berkeley University of California Press 1943 (p 349)

20 BOYLES P W FERGUSON J H and MUEHLKE P H Mechanisms involved in fibrin formation *J Gen Physiol* 34, 493 (1951)

21 SEEGERS W H Quantity of thrombin required to clot heparin plasma mixture *Proc Soc Exper Biol & Med* 51 172 (1942)

22 KAY J H and HUTTON S B JR Studies on an antithrombin 1 The antithrombic activity of alpha tocopherol phosphate *Bull Tulane Medical Faculty* 9 122 (1950)

23 QUICK A J and GROSSMAN A M Nature of hemorrhagic disease of newborn delayed restoration of prothrombin level *Am J M Sc* 199 1 (1940)

24 FERRY J D and SHULMAN S The conversion of fibrinogen to fibrin 1 Influence of hydroxl compounds on clotting time and clot opacity *J Am Chem Soc* 71 3198 (1949)

25 SEEGERS W H and SMITH H P Factors which influence activity of purified thrombin *Am J Physiol* 137 348 (1942)

26 GERBER C F and BLANCHARD E W Effect of certain substances on clotting time *in vitro* *Am J Physiol* 144 447 (1945)

27 ZIERLER K L GROB D and LILIENTHAL J L JR On the antithrombic and antiproteolytic activity of alpha tocopheral phosphate *Am J Physiol* 153 127 (1948)

28 KAY J H *et al* Studies on an antithrombin III A plasma antithrombin test for the prediction of intravascular clotting *Surgery* 28 24 (1950)

29 KAY J H *et al* The prophylaxis of intravascular clotting by the use of alphatocopherol *New Orleans Med & Surg J* 103 116 (1950)

30 KAY J H and BALLA G A Antithromboplastic activity of monosodium alpha tocopheral phosphate and inositol phosphatide *Proc Soc Exper Biol & Med* 73 465 (1950)

31 ASTRUP T and DARLING S Antithrombin and heparin *Acta physiol Scandinav* 5 13 (1943)

32 SYLVÉN B On the cytoplasmic constituents of normal tissue mast cells VIIth International Congress International Society for Cell Biology 1950 (p 49)

33 THOMAS L Studies on intravascular thromboplastic effect of tissue suspensions in mice 1 The reaction of mice to intravenous injections on a sedimentable tissue component *Bull Johns Hopkins Hosp* 81 1 (1947)

34 MACFARLANE R G Action of soya bean trypsin inhibitor as anti thromboplastin in blood coagulation *J Physiol* 106 *104 (1947)*

35 KARRER P and BUSSMAN G *d l* alpha Tocopherol phosphor saure ester *Helv Chim Acta* 23 *1137 (1940)*

36 QUICK A J Normal antithrombin of blood and its relation to heparin *Am J Physiol* 123 *712 (1938)*

37 KAY J H *et al* The prophylaxis of intravascular clotting by the use of alpha tocopherol and calcium *New Orleans M & S J* 103 *116 (1950)*

38 KLEIN P D and SEEGERS W H The nature of plasma antithrombin activity *Blood* 5 *742 (1950)*

39 HURN M BAKER N W and MANN F D Variations in prothrombin and antithrombin in patients with thrombosing tendencies *Am J Clin Path* 17 *709 (1947)*

40 THOMAS L Studies on intravascular thromboplastic effect of tissue suspensions in mice II A factor in normal rabbit serum which inhibits the thromboplastic effect on the sedimentable tissue component *Bull Johns Hopkins Hosp* 81 *26 (1947)*

41 PAULING L *Nature of the Chemical Bond and the Structure of Molecules and Crystals* Second Edition Ithaca Cornell University Press 1940

42 HILDEBRAND J H and SCOTT R L *The Solubility of Non electrolytes* New York Reinhold Publishing Corp 1950

43 EDSALL J T and COHN E J *Proteins Amino Acids and Peptides As Ions and Dipolar Ions* New York Reinhold Publishing Corp 1943

44 BERNAL J D and FOWLER R H A theory of water and ionic solution with particular reference to hydrogen and hydroxyl ions *J Chem Physics* 1 *515 (1933)*

45 HENDERSON L J *The Fitness of the Environment* New York The Macmillan Co 1913

46 BERNAL J D and FANKUCHEN I X ray and crystallographic studies of plant virus preparations I Introduction and preparation of specimens II Modes of aggregation of the virus particles *J Gen Physiol* 25 *111 (1941)*

47 ONSAGER L The effects of shape on the interaction of colloidal particles *Ann New York Acad Sc* 51 *627 (1949)*

48 DERVICHIAN D G Coacervation as phase equilibrium in colloidal systems *Research* 2 *210 (1949)*

49 PAULING L and WILSON E B JR *Introduction to Quantum Mechanics* New York McGraw Hill Book Co 1935

50 ROJANSKY V *Elementary Quantum Mechanics* New York Prentice Hall Inc 1938

51 EYRING H WALTER J and KIMBALL G E *Quantum Chemistry* New York John Wiley & Sons Inc 1944

52 SEXTON W A *Chemical Constitution and Physiological Activity* London Spon 1949 New York Van Nostrand 1950

53 SNELLMAN O SYLVÉN B and JULEN C Analysis of the native heparin lipoprotein complex including the identification of a heparin complement (heparin co factor) obtained from extracts of tissue mast cells *Biochim Biophys Acta* 7 98 (1951)

54 FERGUSON J H Modus operandi of commercial heparin *Proc Soc Exper Biol & Med* 37 23 (1937)

55 ——— Action of heparin serum albumin (crystalline) and salmine on blood clotting mechanisms (*in vitro*) *Am J Physiol* 130 759 (1940)

56 GLAZKO A J and FERGUSON J H Kinetics of thrombin inactivation as influenced by physical conditions trypsin and serum *J Gen Physiol* 24 169 (1940)

57 FERRY J D Consideration of the structure and mechanism of formation of the fibrin clot *Blood Clotting and Allied Problems* Flynn J E Editor Trans Second Conf New York Josiah Macy Jr Foundation 1949 (p 95)

58 WRIGHT I S and HORAN F Zeta potential studies *Blood Clotting and Allied Problems* Flynn J E Editor Trans Second Conf New York Josiah Macy Jr Foundation 1949 (p 141)

THE TRANSITION OF FIBRINOGEN TO FIBRIN

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"Again things which look to us hard and dense must consist of particles more hooked together and be held in union because welded all through with branch like elements

Those things which are liquid and of fluid body ought to consist more of smooth and round elements †

WHEN THROMBIN is added to a fibrinogen solution a dramatic change soon occurs the solution becomes a solid gel This change in proper ties of fibrinogen has a much wider interest than just being an episode in the process of blood clotting This sol gel change can be considered an example of the never ceasing sol gel change in the protoplasm of a living cell It may also give a clue as to how bio logical fibrous structures are built up

The gelatin of fibrinogen occurs at such a low protein concen tration that as Ferry(1) pointed out it has to be assumed that it consists of a three dimensional network of long branching particles One look at an electron microscopic picture of fibrin shows that the fibrin gel is really a network structure It is now known that the long fibrous particles in the fibrin gels are not the result of unfolding of the polypeptide chains of the fibrinogen molecules but rather a polymerization of the fibrinogen molecules to form long fibers

The difference in properties between fibrinogen and fibrin can be considered due to a possible chemical modification brought about by thrombin and as a result of the network structure

The most obvious assumption would be that thrombin as an enzyme catalyzes the combination of fibrinogen molecules so that long fibers are built up

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† Lucretius *De Rerum Natura* Book II

Apart from the very fact that the network structure is the result of thrombin action, no other evidence points toward the correctness of this assumption.

Several observations indicate, however, that thrombin is really not involved in the polymerization process. First, there is always a latency period before the polymerization starts, showing that something else happens before the actual association of the fibrinogen molecules takes place. Second, a clot formed from purified fibrinogen can be easily dissolved in urea or guanidine(2, 3, 4, 5, 6). Fibrin dissolved in urea has the same viscosity as that of fibrinogen in urea. Moreover, our recent light scattering experiments in collaboration with Dr. Steiner have shown that fibrin in guanidine has the same dimensions and *molecular weight* as *fibrinogen*. No doubt these reagents disperse fibrin to the fibrinogen level. The only *difference found up to now is that these dispersed fibrin molecules* have a slightly different isoelectric point.

Yet when urea is dialyzed off, one can get back the clot. It is *known that there are two extreme types of clots*: the *turbid* and the transparent clots(7, 8, 9, 10). Depending on the pH and salt concentration against which it was dialyzed, the turbid or the transparent clot can be reconstituted, which also shows that these two types of clots are interconvertible.

Furthermore, by adding a small amount of serum to the fibrinogen, the thrombin disappears soon after clotting and yet the clot dissolved in urea can be reconstituted.* Thrombin is clearly not involved in the formation of the reconstituted gel structure.

Third, under certain conditions it is possible to show that whereas the action of thrombin manifests itself, there is no gel formation *nor appreciable polymerization*†(11). *At around pH 5 in* about 0.3 M KCl there is no gel formation when thrombin is added, nor even a change in viscosity, whereas *samples taken from the reaction mixture from time to time* and *reneutralized* show that the clotting time becomes shorter and shorter. Even after *twenty four* hours incubation, when the clotting of the reaction mixture upon reneutralization is instantaneous, there is *no change in the sedimenta*

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* The dissolution and *reconstitution of the clot in itself is a remarkable phenomenon*. A highly organized structure can be broken down to the original building stones and will be reconstituted again to an apparently highly organized clot when the ionic milieu is suitable. *Either the geometry of the particles or geometry of the* forces must be responsible for it.

† Unpublished data.

tion pattern of the mixture Our recent light scattering experiments(12) show that at the salt concentration used and at a wide range of pH the weight average molecular weight just before the gel point is about four million and the length of the particle is 2 500 A (see Figure 60) Though the situation in the incubated mixture should correspond to the situation existing just before the gel point (instantaneous clotting) neither the ultracentrifuge pattern viscosity or light scattering reveal noticeable amounts of particles found to exist before the gel point

From all these observations I can draw the conclusion that thrombin is not involved in the polymerization and building up of the gel The role of thrombin seems to be limited to making some alterations on the fibrinogen molecule the only detectable result of which is a slight change in the isoelectric point of the fibrinogen molecule

All the evidences available point to an enzymic nature of thrombin Practically any small amount of thrombin is capable of clotting any amount of fibrinogen Thrombin is either an enzyme coenzyme or a prosthetic group of an enzyme already present in fibrinogen preparations

Three major questions have to be answered if we are to understand what happens when thrombin clots fibrinogen 1 How does thrombin prepare fibrinogen molecules for polymerization? 2 What is the mechanism by which the highly organized fibrin gel is built up? 3 What are the forces connecting the particles?

1 THROMBIN PREPARATION OF FIBRINOGEN MOLECULES FOR POLYMERIZATION

Several lines of approach suggest themselves to answer this question a) Study the differences between fibrinogen and fibrin in order to reconstruct what the action of thrombin was b) Find other substrates for thrombin c) Find other agents that clot fibrinogen producing a gel similar to that of fibrin

a) Differences between fibrinogen and fibrin

Bailey and Lorand independently undertook a comparative amino end group analysis of fibrinogen and fibrin* Both came to the conclusion that while fibrinogen contains no glycine end groups

* Personal communication.

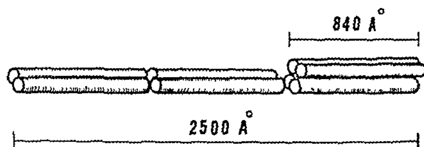


FIGURE 60 Probable configuration of a fibrin particle just before the gel point

fibrin contains four or five such end groups per fibrinogen molecule (Mw = 550 000) Lorand points out that although proteolytic impurity *in the thrombin preparation cannot be ruled out it is not* very likely that the increase is due to proteolysis Bailey points out that thrombin has no such end group liberating effect on other *proteins thus it is very specific for fibrinogen*

It is known that fibrinogen contains 1 2 per cent carbohydrate (13) expressed as galactose In my experiments on several purified fibrinogen preparations I found 1 29 per cent carbohydrate *ex* pressed as galactose I also found that fibrin always contains less carbohydrate about 15 to 20 per cent less than fibrinogen According to these data 1 molecule of fibrinogen (Mw = 550 000) *contains* about 39 moles of galactose and fibrin 5 to 6 moles less It is possible that after clotting glucoprotein impurity with higher carbohydrate *content than fibrinogen remains in the supernatant and this is the* explanation for finding less carbohydrate in fibrin

The isolation of the substance appearing after clotting is in the preliminary stage and no conclusion can yet be drawn The fact that incubation of fibrinogen with carbonyl reagents like phenylhydrazine semicarbazide hydroxylamine progressively inhibit the clottability *of fibrinogen suggests that the carbohydrate in fibrino* gen might be involved (Here again the possibility that these re agents activate fibrinolytic activity cannot be completely excluded)

It would be tempting to imagine that a small polysaccharide *is split off* from fibrinogen by the action of thrombin much more proof is needed however until it can be definitely established whether the carbohydrate complex *of fibrinogen is in any way* involved in the clotting process

b) Other Substrates for Thrombin

No substrate other than fibrinogen is yet known for thrombin

c) *Other Agents That Clot Fibrinogen*

To find other agents we have to look for hydrolytic enzymes* Among the hydrolytic enzymes papain(16) and certain snake venoms(17) are known to clot fibrinogen

The clotting of fibrinogen by papain would be especially interesting in this connection if it could be shown that it was the proteolytic enzyme itself that clots fibrinogen and that the clot formed is similar to the clot formed by thrombin However one has to be sure that the papain acts directly on fibrinogen and not by converting the possible prothrombin contamination present in fibrinogen preparations to thrombin

There are many indications that papain produces a clot very similar or even identical to that of thrombin The papain clot under the microscope(18) or electron microscope appears to be the same as the thrombin clot The kinetics of the formation of the turbid clot seems to follow the type of a first order reaction When the polymerization of fibrinogen is followed by light scattering methods it appears to be similar to that brought about by thrombin These observations indicate that the mode of action of papain is similar to the mode of action of thrombin

The possibility that the papain does not act directly on fibrinogen but through prothrombin can almost certainly be excluded Papain clotted purified fibrinogen just as well even when there was no sign that fibrinogen contained prothrombin as an impurity

If all the above mentioned points are accepted there remains the possibility that even in crystallized papain preparations a thrombin like impurity (19) causes the clotting In this connection I would like to mention a few experiments which strongly support the idea that it is the proteolytic enzyme papain that produces the clot

It is known that papain can be activated by SH substances and by KCN I found that the clotting activity becomes activated to the same extent as the proteolytic activity

Under the experimental conditions used in this work KCN activated proteolytic activity (studied by the clot dissolving activity)

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* Oxidation or reduction does not seem to be involved in the thrombin action Thrombin clots fibrinogen in the absence of oxygen in vacuum or CO atmosphere We made numerous attempts to test the hypothesis of Lyons(14) according to which SH groups are involved in clotting On testing various kinds of SH reagents we came to the conclusion that SH groups are not involved in the clotting(15)

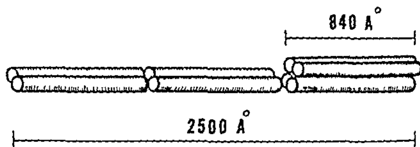


FIGURE 60 Probable configuration of a fibrin particle just before the gel point

fibrin contains four or five such end groups per fibrinogen molecule (Mw = 550 000) Lorand points out that although proteolytic impurity in the thrombin preparation cannot be ruled out it is not very likely that the increase is due to proteolysis Bailey points out that thrombin has no such end group liberating effect on other proteins thus it is very specific for fibrinogen

It is known that fibrinogen contains 1 2 per cent carbohydrate (13) expressed as galactose In my experiments on several purified fibrinogen preparations I found 1 29 per cent carbohydrate expressed as galactose I also found that fibrin always contains less carbohydrate about 15 to 20 per cent less than fibrinogen According to these data 1 molecule of fibrinogen (Mw = 550 000) contains about 39 moles of galactose and fibrin 5 to 6 moles less It is possible that after clotting glucoprotein impurity with higher carbohydrate content than fibrinogen remains in the supernatant and this is the explanation for finding less carbohydrate in fibrin

The isolation of the substance appearing after clotting is in the preliminary stage and no conclusion can yet be drawn The fact that incubation of fibrinogen with carbonyl reagents like phenylhydrazine semi carbazide hydroxylamine progressively inhibit the clottability of fibrinogen suggests that the carbohydrate in fibrinogen might be involved (Here again the possibility that these reagents activate fibrinolytic activity cannot be completely excluded)

It would be tempting to imagine that a small polysaccharide is split off from fibrinogen by the action of thrombin much more proof is needed however until it can be definitely established whether the carbohydrate complex of fibrinogen is in any way involved in the clotting process

b) *Other Substrates for Thrombin*

No substrate other than fibrinogen is yet known for thrombin

2 THE MECHANISM OF BUILDING UP THE GEL STRUCTURE

Electron microscopic pictures (21 22) show that the fibrin gel is a three dimensional network of branching bundles The diameter of these bundles varies from a few hundred Å to 2000 Å The most intriguing feature of these bundles is regular cross striations about 250 Å apart There are indications that within this regularity even finer regularity exists

No satisfactory explanation has been found yet for the formation of these regular structures There is no doubt that the building stones for these structures are the fibrinogen molecules only slightly modified by thrombin

The length of the fibrinogen molecules is estimated to be around 700 Å and the molecular weight around 500 000 Oncley Scatchard and Brown(23) studying human fibrinogen (sedimentation constant osmotic pressure viscosity) arrived at a molecular weight of about half a million Edsall Foster and Scheinberg(24) estimated a length of 700 Å from double refraction of flow measurement Nanninga(25 26) studying the osmotic pressure and viscosity of bovine fibrinogen arrived at a molecular weight of 440 000 and a length of 740 Å

Our recent light scattering measurement made upon six different samples of fibrinogen* at pHs 8 4 6 98 and 5 gave a molecular weight of 540 000 ± 5 per cent and a length of 840Å ± 5 per cent on the basis of a rod

At high pH (8 4) and ionic strength (0 35) the increase in average molecular weight with time as a result of thrombin action

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The fibrinogen for these experiments was prepared as follows 4 gm fibrinogen (Armour bovine plasma fraction I) is dissolved (gentle stirring with a glass rod) in 200 ml phosphate buffer (20 ml KH PO M/2 + 10 ml Na HPO M/2 in 200 ml H O) After dissolution (there may remain some undissolved particles) 200 ml H O is added to the solution and 5 drops of N HCL The solution is left standing overnight in a refrigerator The next morning there is a sediment at the bottom of the vessel Without stirring the solution is filtered through a Whatman folded filter paper No 12 (other paper probably will do) I use two filter papers filtering about 200 ml through each The result is about 370 ml water clear solution To this solution 130 ml saturated (NH)SO is added in small portions under gentle stirring The white flocculent precipitate is centrifuged down (Part of the precipitate is packed on the top and lifted out with a glass rod) The precipitate is dissolved in 60 ml 0 3 M KCl solution (during dissolution a few drops of a 3 per cent NH or NaOH is added to bring the pH around pH 7 2) This solution is dialyzed against 1 liter 0 3 M KCl in the cold for two to three days changing the KCl solution several times (Negligible reaction with Nessler reagent at the end of dialysis) The solution is water clear and contains around 12 mg clottable protein per ml and the purity is about 95 per cent (according to my method of estimating the purity)

about thirty times and the clotting activity twenty six to twenty eight times

It has also been found that varying amounts of $KMnO_4$ destroyed proteolytic activity of papain to the same extent as the clotting activity was destroyed

These observations very strongly suggest that it is the proteolytic enzyme papain which clots fibrinogen so the bond in the fibrinogen molecule attacked by thrombin must be such that it can be attacked by papain

Papain splits peptide bonds and amides like hippuryl amide Some of the proteolytic enzymes even split esters of amino acids There is certainly a limited number of bond types the papain can split

In this connection the experiment of Guest and Ware(20) offers special interest They found that a clot produced by thrombin slowly dissolves and that this dissolution is affected by heat to the same extent as is the clotting activity of thrombin Though the clot dissolution is usually attributed to plasmin it is not altogether impossible that thrombin itself can dissolve the clot

Then if we compare thrombin with papain and trypsin we might say thrombin is very active in clotting but very weak in proteolysis Papain is active in clotting and also active as a proteolytic enzyme Trypsin is very active as proteolytic enzyme but very weak in clotting effect on fibrinogen (Trypsin never clots fibrinogen but very quickly digests fibrinogen or fibrin)

Now if thrombin splits a bond like a peptide bond the cleavage products must be a small molecule and a practically unchanged fibrinogen molecule If the bond was a combination of amino groups and carboxyl groups one would observe no pH change during clotting but if the bond was a particular ester of an amino acid a change in the pH with clotting ought to have been observed As far as I know it is generally accepted that no pH change occurs during clotting (this question needs more careful study however) It thus seems likely that the bond split by thrombin is a kind of peptide bond involving amino groups and carboxyl groups and if we agree that fibrin has a changed isoelectric point (toward neutral pH) we might conclude that the amino group part of that particular bond remained on the fibrinogen Thus if the action of thrombin really was such as pictured above there ought to have been an increase in the number of amino groups in the fibrin

the reaction but actually depolymerize stepwise the polymer by successive addition of permanganate

These experiments indicated that first the end to end associations were broken by the permanganate then lateral associations and finally particles with the size and shape of fibrinogen molecules remained in solution It seems that the bond connecting the particles side by side is stronger than the bond of end to end associations

No definite conclusion can be drawn from these experiments about the nature of the bonds holding the particles together I believe however that we have to look for some specific bond Whether such a bond or bonds involve the participation of amino groups and carbohydrate groups cannot yet be stated The above circumstantial evidence seems to point in this direction

I would like to point out that the clot formed in blood plasma is different from the clot formed from purified fibrinogen

The plasma clot is not soluble in urea It has been found that insoluble clot from purified fibrinogen can be obtained if the fibrinogen clots in the presence of calcium ions and serum(2 3 5) In a recent report Lorand(5) points out that there is another difference between the two types of clots fibers of the urea insoluble fibrin change more easily from alpha to beta configuration (Astburys terminology) on stretching than fibers of the urea soluble fibrin

I S Wright Thank you Dr Laki Dr Waugh will you carry on from here?

DISCUSSION

Waugh Dr Laki has outlined the important points which must be considered in discussing the way thrombin and fibrinogen interact the bonds which are involved in the linkage of these units and the way in which the final clot structure is developed

Actually I find myself very much in agreement with the ideas which he has presented Minor points to which answers could not be given could be raised As examples Are the bonds which are attacked by papain and thrombin in the fibrinogen molecule the same the specific mechanisms by which fibrin strands form and so on?

follows a sigmoidal curve The curve levels off at around the gel point and the dissymmetry does not increase beyond the limiting value of a rod At this stage the molecular weight is about eight times that of fibrinogen and the length is almost three times as great It seems that the gel is built up from these units

At the other extreme low pH (6 35) and low ionic strength (0 24) the picture is different Lateral aggregation clearly pre dominates over the end to end aggregation At the time when the average molecular weight is double that of fibrinogen the length is still the length of fibrinogen It seems that under these condi tions (favoring the turbid clot) the gel strands are predominantly built up from lateral association of the fibrinogen rods

I believe that these experiments clearly show that the mechanism of the formation of the strands in the gel is that first bigger units with end to end and side by side association are built up and then these associate to form the gel It will be important to find out whether the formation of the bundles from these units is in the direction of the end to end or to the side by side aggregations We are attempting to get an answer to this question by studying the spectrum of oriented fibrin films in polarized U V light

3 FORCES HOLDING THE PARTICLES TOGETHER

It is fairly clear that before gelation of fibrinogen there are formed large polymer particles made up from unit particles associated end to end and side by side The important question is What are the forces that hold these particles together?

The bonds are certainly such as to break in the presence of concentrated urea

Experiments to stop clotting or destroy clottability with formalde hyde or quinone suggest that amino groups are involved in the bonds between particles(27) There are not many reactions in which amino groups can take part in a manner that urea would be able to disrupt the bond

Most likely such reactions would be the reaction of amino groups with carbonyl groups such as formaldehyde or aldehyde groups of carbohydrates

Our light scattering experiments in which we stopped the poly merization with $KMnO_4$ showed that it is possible not only to stop

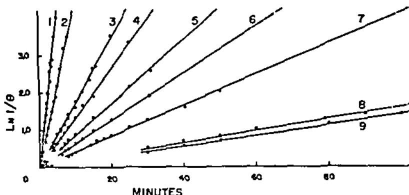


FIGURE 61 Linear portions of reaction curves for Ø = 0 185 mg CN per ml. Thrombin concentration in units per ml were curve 1 0 454 curve 2 0 227 curve 3 0 091 curve 4 0 068 curve 5 0 045 curve 6 0 023 curve 7 0 018 and curves 8 and 9 0 009 The temperature was 22 7 C

When one varies the fibrinogen concentration as shown in Figure 62 a series of straight lines is obtained again on the basis of a first order plot Since these lines have different slopes the reaction is not predicted on the basis of an equation similar to Equation B

$$-\frac{d\phi}{dt} = k\ (Th_o)(\phi) \qquad (B)$$

An analysis of the curves has shown that they can be represented by Equation C which is

$$-\frac{d\phi}{dt} = k\frac{Th_o K}{K + \phi}\phi \qquad (C)$$

where $kK = 0\ 472$ and the equilibrium constant $K = 0\ 051$ K is given by

$$K = \frac{(Th)(\phi)}{(Th\phi)} \qquad (D)$$

where ϕ is the initial fibrinogen concentration

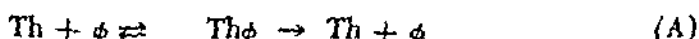
The complex $(Th\phi)$ given in Equation D apparently is not an active complex Thus Equation D is reasonably explained by assuming that thrombin and fibrinogen react and form an inactive complex thus fibrinogen acts as its own inhibitor The changes in slope shown in Figure 62 are due to this inactive complex formation Equation D also suggests that fibrin combines with thrombin to the same extent as fibrinogen The foregoing does not exclude

*Figures 61 and 62 printed by permission from *Journal of Physical and Colloid Chemistry* (In press)

I would like to present information which Mrs Betty Livingstone and I have obtained by studying the interaction of Armours thrombin and Fraction I* and our thoughts on clotting mechanism and the relationship of clotting time to clot structure The results of these investigations are published or will be published soon (28 29)

In considering clotting in solutions of fibrinogen and thrombin we have worked at a pH of 6 85 and an ionic strength of 0 15 throughout I think that Dr Laki rightly emphasized the importance of viewing fibrinogen activation as involving a very light structural alteration in the fibrinogen molecule and certainly a minor chemical alteration

I feel that we can assume that the interaction of thrombin and fibrinogen involves the formation of some sort of an active complex according to Equation A



$$Th + \phi \rightleftarrows Th\phi \rightarrow Th + \phi \qquad (A)$$

where Th represents thrombin (N I H units per ml) and ϕ fibrinogen (mg clottable nitrogen per ml) The complex $Th\phi$ represents an active complex This will then decompose into activated fibrinogen ϕ and thrombin The activated fibrinogen will then polymerize to give fibrin ϕ will be used to represent activated fibrinogen and fibrin

It was Equation A which occupied our interest at the start I would like to show you the results of the kinetic studies Aliquots of fibrinogen and thrombin solutions were mixed At various times the clot was compacted mechanically and formaldehyde was added to stop the reaction The compactable fibrin was centrifuged off and the supernatants analyzed for their content of unchanged fibrinogen and noncompactable fibrin For convenience the results are plotted as though a first order reaction were involved thus $\phi / \phi = \ln 1/O$

Figure 61 shows a series of curves obtained by holding the fibrinogen concentration constant and varying the thrombin concentration Over a fiftyfold range in thrombin concentration one gets a series of straight lines which go essentially to completion and which have initially nonlinear portions which are represented by dotted lines I will return to this shortly The slopes of all lines of Figure 61 are proportional to the concentration of thrombin in solution

* This work was supported by a grant in aid from the Chemical Research and Development Department of Armour and Company Chicago Illinois

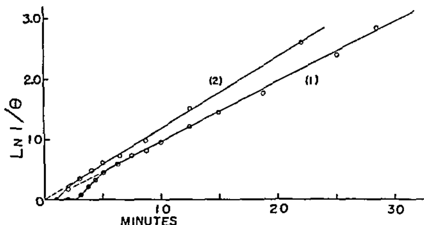


FIGURE 63 A typical reaction curve Ø = 0 18 mg CN per ml and Th = 0 045 units of thrombin per ml (curve 1) Curve 2 represents the same reaction run in the presence of 1 54% purified gum acacia Reprinted from article by D F Waugh and B J Livingston *Science* 113 *121 (1951)* Also by permission from *Journal of Physical and Colloid Chemistry* (In press)

fibrin and noncompactable fibrin In other words we are compacting only a certain portion of the total fibrin present

The results so far indicate that two of at least four possible activation mechanisms are improbable The first mechanism would be one in which a thrombin activation was required at each end of the fibrinogen molecule and that these activations were essentially separate events This is essentially a double activation theory One would get entirely different reaction curves on this basis I think that we can discard the possibility that two interactions between a single fibrinogen molecule and thrombin are required for activation

Kinsely What type of curve would you get?

Waugh Plotted as a first order reaction an exponential curve would be obtained It would have a long lag period and then would rise linearly On extrapolation the curve would intersect the time axis at about 0 2 of the value which the curve achieves at ln 1/O = 2 0 a circumstance which is entirely outside of the experimental error

The second type of thrombin fibrinogen interaction visualized as leading to activation is one in which a single event only is necessary but activated fibrinogen molecules combine with native fibrinogen molecules The clot would then be a mixture of activated and native fibrinogen molecules One would expect fibrin formation to

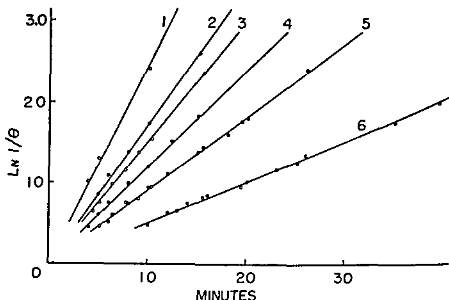


FIGURE 62 Linear portions of the reaction curves for Th = 0.0454 units of thrombin per ml The fibrinogen concentrations were in mg CN per ml curve 1 0.036 curve 2 0.075 curve 3 0.088 curve 4 0.11 curve 5 0.182 and curve 6 0.355 The temperature was 22.7 C

formation of a true active complex Indeed it must form It simply means that one cannot alter the concentrations of thrombin and fibrinogen sufficiently to make an active complex observable *kinetically*

In Figure 63 is shown a general reaction curve in which the entire course of the reaction is depicted Referring to curve 1 one can see the initial nonlinear portion It is this particular portion of the curve which becomes important in considering such matters as clotting times *Figure 63 shows an initial lag phase during which time* sufficient fibrin has formed to establish a compactable clot During this time the suspension contains free fibrin fibrils and activated fibrinogen which has not yet hooked up with other molecules Therefore we find no compactable fibrin After the initial lag phase compactable fibrin appears and a certain amount of fibrin can be removed A sigmoid portion joins the axis and the linear portion of the curve Let us examine a point which is on the non linear rise Let us first consider that the extrapolated portion of the curve predicts the appearance in solution of activated fibrinogen whatever its physical state Therefore a point on the sigmoid portion of the curve must *represent the difference between compactable*

chanical strength to resist deformation One can use the same line of reasoning in considering clotting times which are measured on the basis of the first appearance of fibrin strands in the agitated tube However at the clotting time T there is present not only the cross linked structure but a complement of activated fibrinogen molecules dimers trimers and short fibrin strands which exist free in solution and do not contribute to the rigidity of the clot

Referring to the complete reaction curve shown in Figure 63 it has been found that clotting takes place just after the reaction curve leaves the abscissa At this point the bulk of the total fibrin which includes all products beyond activated fibrinogen is in the free state and is not in the compactable clot structure A correlation between our kinetic curves and the corresponding clotting time curves has been obtained Equation C predicts the appearance of activated fibrinogen in solution The total amount of fibrin ϕ = $\phi - \phi$ including compactable and noncompactable fibrin which is present at the instant of clotting may be calculated by substituting measured clotting times in Equation C In this way Equation E is obtained

$$\phi = \frac{\phi - 0\,11}{3\,8 - \mathrm{Ln}\ \ \mathrm{Th}_o} \qquad \text{(E)}$$

Equation E shows that the quantity of total fibrin necessary to establish clotting actually increases both with increasing fibrinogen and thrombin concentrations The quantity of fibrin necessary to establish clotting is therefore a variable

If Equation E and Equation A are combined using T (clotting time) we obtain Equation F

$$T = \frac{0\,051 + \phi}{0\,452\ \mathrm{Th}_o}\ \mathrm{Ln}\ \frac{\phi\,(3\,8 - \mathrm{LnTh}\ )}{\phi\,(2\,8 - \mathrm{LnTh}\ ) - 0\,11} \qquad \text{(F)}$$

Figures 64 and 65 show T vs thrombin concentration (Figure 64) and T vs fibrinogen concentration (Figure 61) The solid lines are calculated according to Equation F the points are averages of several determinations

Kinsely Please sir what system is this? Is this thrombin on fibrinogen?

Waugh Yes

Knisely It is not prothrombin?

Waugh No this is thrombin Prothrombin is not involved

be a function of the square of the initial fibrinogen concentration I do not think that our reaction curves are consistent with this interpretation

We are then led to the assumption that a single collision between a thrombin molecule and a fibrinogen molecule is necessary to *achieve activation We again have two choices in the matter From* the physical nature of the clot one would expect that the fibrinogen molecules are bonded essentially either endwise or sidewise If they had equal binding capacities over their surfaces one would get balls of fibrin and not elongated strands Dr Laki has already discussed these two possibilities Let us consider just one of them endwise bonding

Let us designate the ends of the fibrinogen molecule as 1 and 2 It is assumed that the thrombin molecule activates end 1 of each fibrinogen molecule which then becomes reactive and combines with end 2 of another molecule A propagated clot structure could be obtained in this fashion It is also assumed that the unactivated end 1 will not combine with unactivated end 2 or with unactivated end 1 This type of mechanism will certainly account for all of the kinetic information which we or others have obtained

There is another possibility which has been intriguing This possibility is that a single activation (collision between thrombin and fibrinogen) is required but that the two ends now become cohesive It has been shown that a fibrinogen molecule is about 700 Å long Assuming that the thrombin molecule activates by combination at the center the new bonds then appear at the two ends If this is the case some sort of a wave of chemical activity would have to be propagated along the fibrinogen molecule in the two directions in order to account for activation The extrapolation is clear If a wave of activity can go for 350 Å, it can go for 1000 or 100 000 Å Thus fibrinogen activation may have a direct connection with other more complicated biological systems which show propagated activity We have not been able to separate these last two mechanisms Either would be quite sufficient to account for the experimental results

I would like to turn our attention to the construction of the clot a subject which poses another kinetic problem When does a solution gel or when is a clotting time measured? Gelation occurs when a cross linked network of fibrin strands extends throughout each unit volume and when this linked system has sufficient me-

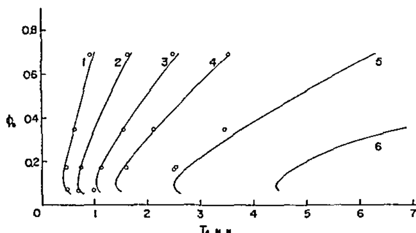


FIGURE 65 Clotting time in minutes as a function of the initial fibrinogen concentration in mg C N per ml The curves and corresponding thrombin concentrations in N I H units per ml are (1) 0 454 (2) 0 27 (3) 0 136 (4) 0 09 (5) 0 045 and (6) 0 023

crease If it is changing more slowly than the rate then clotting time decreases

The second possible effect which might be operating is as follows If one visualizes the clot as being constructed of close packed tetrahedra in which a common edge represents a fibril then if the fibril maintains a constant axial ratio (length of tetrahedran edge to fibril diameter) the amount of fibrin necessary to establish a clot is independent of the size of the tetrahedra On the other hand if the ratio of length of tetranedran edge over the fibril diameter decreases as the fibril length becomes shorter (the diameters are relatively larger in a fine clot than a coarse clot) it will take more fibrin to establish clotting in the fine clot than in the coarse clot This also may be a partial explanation for changes in clotting time observed when fibrinogen and thrombin concentrations are altered

There is an additional item If one adds acacia to the systems described the nonlinear portion of the reaction curve is greatly shortened The point at which the reaction curve leaves the abscissa is shifted toward decreasing times with increasing acacia concentrations It clearly can never attain zero since it always requires a certain amount of fibrin to establish clotting The rate of appearance of activated fibrinogen under the same circumstances is changed by a relatively small amount possibly 10 per cent Curve 2 in Figure 63 was run in the presence of 1 5 per cent

As has been observed the clotting time increases with increasing fibrinogen concentration There is a T_c minimum in the clotting time curve As the fibrinogen concentration goes below a certain value clotting time again increases The experimental points are in reasonable agreement with the calculated curves

The explanation for variations in clotting time obtained when one varies the fibrinogen and thrombin concentrations indepen

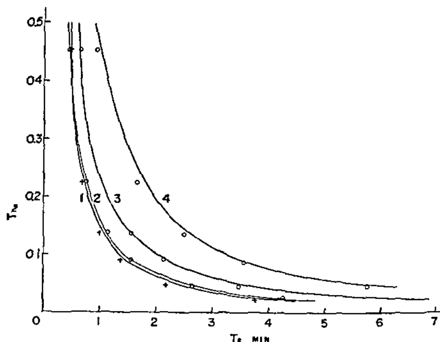


FIGURE 64* Clotting time in minutes as a function of thrombin concentration in N I H units per ml of final solution The curves and initial fibrinogen concentrations in mg CN per ml are (1) 0 07 (2) 0 17 (3) 0 345 and (4) 0 68

dently are to be sought in the relative balance between change in rate and change in ϕ As one increases thrombin concentration for example the reaction velocity increases more rapidly than ϕ and clotting time decreases The opposite is the case when the initial fibrinogen concentration is increased

There are two sources I think to which a change in ϕ might be attributed The first is the ratio between clot fibrin and potential fibrin (or free fibrin) that is fibrin which remains in suspension If this ratio alone assuming a constant amount of clot fibrin is changing faster than the reaction rate the clotting time must in

*Figures 64 and 65 are reprinted from article by D F Waugh and B J Livingston *Science* 113 121 (1951)

point of linearity the total fibrin is distributed between clot fibrin and free fibrin

Flynn I wasn t clear whether that was an experimental determination or deduced

Waugh It is deduced from two things the rate curves and the clotting time measurements

Tocantins May I say something in connection with the possible application of these concepts to the interpretation of the clot accelerating effect of moderate dilutions of blood? It is difficult to see what bearing the concentration of fibrinogen has on this effect since if we use purified beef fibrinogen as supplied by Dr Seegers *as a diluent instead of 0 85 per cent NaCl the curves that result* are alike and that is maintaining the same fibrinogen concentration all along

Waugh Constant?

Tocantins Yes Prothrombin solutions are the only thing that alter the course of the dilution curve Diluting the plasma with prothrombin solutions will alter the parabolic course of the dilution curve The curve will flatten out on the left because prothrombin concentration is not being reduced The reason why the left limb of the curve rises when saline is used as a diluent is because of diminution of prothrombin in the clotting mixture Maintaining fibrinogen concentration constant however does not alter the shape of the dilution curves

Waugh Actually there is no way of extrapolating directly from the experiments which we have done into others Each system is a system on its own and must be analyzed separately

Kniselg The system as you said applied to the kinetics in the change between thrombin and fibrinogen?

Waugh Yes Of course we want to study prothrombin activation

Seegers I wish that Dr Waugh would try platelet extractions having in mind particularly the accelerated factor we found in platelets that aids the interaction of thrombin and fibrinogen I would be very interested to know whether it behaves in much the same way as acacia does In the experiments that we do we cannot tell any difference but with your refined techniques you should be able to tell whether the kinetics are the same or not

gum acacia The reaction rates thus differ by 10 per cent but clotting times change by a factor of approximately three

We have considered the consequences of this observation Assume a certain rate of appearance of activated fibrinogen dependent in a given system on the conversion of prothrombin with all of the factors that affect this conversion

A substance is added to the system which has an accelerating effect in the sense that it shortens clotting time The interpretation of the accelerating effect is not clear The substance could a) increase the rate of conversion of prothrombin to thrombin b) decrease the nonspecific combination of thrombin and fibrinogen thus increasing the concentration of free thrombin c) shift the ratio between clot fibrin and free fibrin or d) increase the average axial ratios of fibrils involved in the structural or clot fibrin

Knisely Can t that be considered an acceleration?

Waugh It is an acceleration yes but the basic question is one of interpretation I am now getting into very unsafe ground so far as I am concerned Suppose that one is going to evaluate an inhibitor a clinical agent on the clotting time basis If the inhibitor acts on prothrombin conversion that is an entirely different matter than if the inhibitor acts in the manner that it decreases compactable clot formation There are clearly a large number of possibilities all along the line

In listening to many of the remarks which have been made about the effects of reagent concentration and the addition of globulins and other colloid molecules to the clotting system I wondered whether some of these effects might not be due to a shift in the structure of the fibrin clot itself rather than to a rate change at some point in the system

Flynn How do you measure the amount of activated fibrinogen Dr Waugh?

Waugh We actually do not calculate the distribution of total fibrin between free fibrin and clot fibrin We calculate the amount of activated fibrinogen which has appeared in the system on the basis of the rate Equation C The point at which linearity is established we interpret as the point at which the concentration of activated fibrinogen and the content of small fibrin strands is small and can be neglected The linear portion of the curve then actually follows the appearance of activated fibrinogen Below the

Even when fibrin bundles are many hundreds or even more than a thousand Angstroms in width nevertheless the alternating dark and light bands run uniformly across the entire width of the bundle Thus if there is lateral aggregation of the elements of the primary network it involves fitting of the different elements side by side into a pattern in which they are held fixed with a high degree of precision

This recalls the formation of collagen fibers by reprecipitation from collagen solution as shown by the studies in F O Schmitt s laboratory at Massachusetts Institute of Technology

Another point that I would like to bring up is the fact that Dr A G Loewy in our laboratory has lately been testing the solubility of fibrin in urea Our preparations do give results fully in accord with those that Lorand and Laki have reported

Lórand has lately reported(5) that the serum factor which with calcium causes the fibrin clots to be insoluble in concentrated urea remains with the fibrinogen when one precipitates plasma with ammonium sulphate in the usual fashion However it can be separated by a subsequent precipitation of the fibrinogen at pH near 5 2 the urea insolubility factor remaining in the supernatant solution Loewy s results using materials separated by low temperature alcohol precipitation run very closely parallel with Lorand s Human Fraction I which is obtained from plasma by precipitation with alcohol (8 to 10 per cent at −3 C) contains about 65 per cent of the protein present as fibrinogen Fraction I forms a clot which is insoluble in 30 per cent urea in the presence of small amounts of calcium ion However a subfraction of much higher purity known as Fraction I 2 has been prepared(30) The process involved a precipitation of Fraction I at pH near 5 1 as the first step and Lorand s work therefore suggested that the urea insolubility factor should be removed at this stage Loewy has indeed found that Fraction I 2 gives a clot with thrombin which dissolves very rapidly in 30 per cent urea even in the presence of calcium ion He has also tested the effect of other ions on the clotting of total Fraction I to see whether they could substitute for calcium Magnesium strontium and barium apparently could not substitute for calcium Strontium may have had a trace of activity but magnesium and barium were definitely inert

I should like to present certain studies on the clotting of fibrinogen (Fraction I) by thrombin carried out by Dr Walter Lever

Jaques May I ask how the fibrin was measured?

Waugh At a certain time the system is compacted rapidly and the clotting reaction is stopped with formaldehyde The compacted fibrin is then centrifuged off and the supernatant analyzed by ultra violet absorption One can take the compacted fibrin and analyze it also

The absorption of the supernatant is corrected for the quantity of nonclottable protein and the residual thrombin

Jaques The reaction is actually frozen by adding formaldehyde?

Waugh Yes

Edsall I was glad to see that Dr Laki s measurements on the size and shape of the fibrinogen molecule are of the same order of magnitude as those that we had obtained by quite independent measurements(24) and that Nanninga(25) had also found inde pendently of us Cecil Hall(22) in his studies with the electron microscope found that a considerable proportion of the fibrinogen molecules in his preparations had a molecular length of the order of 700Å and that the molecular width – too narrow to measure accurately – was compatible with the value of about 38 Å which Nanninga and we had calculated However Hall found large dis tributions of different molecular sizes in his preparation He ob served some short molecules – about 300 Å – and others as long as 1100 Furthermore these molecules did not look like simple rods Instead they looked rather like a string of beads in his pic tures and the different sized molecules seemed to differ primarily with respect to the number of beads that were in the string

Just how to interpret these very important observations I do not know Certainly it is always possible that in the process of prepar ing the materials to examine them in the electron microscope there may have been breakdown and alteration of molecules as they existed in solution So I do not regard the electron microscope as an ultimate authority

Another major problem raised by the electron microscope studies is that of the origin of the striations that appear in fully developed fibrin(21 22) The pattern of the striations is very regular with a repeat period of about 230 Å – somewhat variable according to Hall – but in the fibrinogen molecule or in the very first stages of the formation of fibrin strands there did not appear to be any obvious striations The striations seemed to be created in the process of forming the fibrin clot out of the fibrinogen

TABLE XXVI

Modification of the Clotting Process by Variation of pH and by Addition of Various Reagents

Human Fibrinogen (Fraction I)

E p No	Added Reagent X	Conc in mol/l		pH	t_{50} (min) 50% fibrin time	F_1 fibrin after 1 hour	F_{24} 24 hour yield	Δ ′ after			$\frac{\Delta}{F_1}$	$\frac{\Delta}{F_{24}}$
		Na+Cl−	X					10 min	1 hr	24 hrs		
1	HIm+Cl−	0 12	03	7 3	6	102	110	09	10	12	10	11
2		0 12	03	7 0	7	101	103	24	29	31	29	32
3		12	03	6 7	7	92	110	38	64	74	69	67
4		12	03	6 4	12	90	102	32	68	85	76	83
5	"	12	03	6 3	16	82	100	21	62	85	76	85
6		12	03	6 1	30	69	93	05	50	92	72	99
7	"	12	03	5 8	200	14	95	—	—	—	—	—
8	"	135	015	6 3	14	87	100	23	72	99	83	99
9*	Guan+Cl−	085	050	6 3	45	56	88	02	24	92	43	1 05
10*	"	060	075	6 3	1000	0	55	00	00	69	—	1 25
11*	"	060	075	6 3	400	4	76		High Turbidity			
12*		060	075	7 3	12	92	95	28	58	77	63	83
13*		085	050	6 3	200	20	85		High Turbidity			
14	Na+Cl−	15	—	6 2	10	88	100	40	84	1 02	95	1 02
15	"	30	—	6 2	80	46	92	01	16	61	35	66
16		45	—	6 2	360	15	64	00	04	28	27	44
17	Ca++(Cl−)$_2$	015	0 045	6 2	15	88	108	03	50	1 36	57	1 26
18		015	095	6 2	150	0	106	00	01	34	—	32
19	"	015	145	6 2	1200	0	52	00	00	03	—	06

All solutions contained Fraction I at a total protein concentration of approximately 1 to 1 1 gm per l the fibrinogen concentration was approximately 0 6 to 0 7 gm per l the concentration of human thrombin in the clotting process was 0 1 unit per ml

HIm+Cl− denotes imidazole hydrochloride The solution containing this reagent also contained the basic form of imidazole in a concentration sufficient to give the desired pH

Note that the total ionic strength in solutions 17 18 19 was 0 15 0 30 and 0 45 respectively These solutions are therefore respectively comparable with the sodium chloride solutions of experiments 14 15 and 16

The symbol t_{50} denotes the time required to give half the maximum yield of fibrin this maximum being taken as the amount of fibrin formed under standard conditions at the end of 24 hours

* The solutions in experiments 9 to 13 inclusive contained HIm+Cl− (0 015 M) in addition to guanidine hydrochloride (Guan+Cl−) and sodium chloride Thus the total ionic strength in all these solutions was 0 15 M

and me. They have been briefly referred to in earlier conferences by Dr. Ferry and by me, and a more detailed description is now in press(31). I mention this work here chiefly to indicate the extreme sensitivity of the second phase of the clotting process to small variations in the concentrations of many ions and neutral molecules.

The procedure closely followed that developed earlier by Ferry and Morrison(9, 32). In nearly all the experiments we kept to a constant concentration of fibrinogen — approximately 0.7 gm. per l. — and of thrombin (0.1 unit per ml.) in the clotting mixture. Standard conditions for clotting were taken as 0.15 M sodium chloride solution at pH 6.3, at room temperature (approximately 23°C). A control run was made under standard conditions each time a new experiment was set up to study the effects of adding some other substance to the clotting mixture. For the control, as well as for the experimental solution, seven or eight points on the fibrin formation curve were obtained by determining the weight of the washed and dried clot at various intervals of time after mixing the thrombin with the fibrinogen solution. The weight of the clot formed in the control experiment at the end of twenty-four hours was taken as defining the amount of fibrinogen originally present, and fibrin yields were expressed with reference to this value(32). Note that we were working with Fraction I, not with purified fibrinogen; for the most part it was human Fraction I, but in a few experiments it was bovine. I should add that virtually all of the experimental work is due to Dr. Lever, who has also contributed a great deal to the interpretation of the results.

First, as to the effects of pH and ionic strength, some representative data are given in Table XXVI. The pH was controlled with imidazole buffer at low concentration.* When pH was the variable studied, the total ionic strength was always held at 0.15 with sodium chloride. The turbidity, τ, was determined in a 1 cm. cell with a Beckman spectrophotometer at 350 mμ — a wavelength at which fibrinogen does not absorb light, but only scatters it.†

* At high concentration (0.15 M and above) imidazole buffer is a strong inhibitor of clotting, but at 0.03 M and below, the solutions containing imidazole buffer behaved indistinguishably from the control sodium chloride solutions at the same pH and total ionic strength.

† τ is defined as log (I_0/I), where I_0 is the intensity of the incident, and I of the transmitted light, for a 1 cm. path in the medium. The turbidity τ as defined by Debye and used in most modern work on light scattering is τ multiplied by 2.303 (natural logarithms). The quantity τ, as defined here, is identical with the opacity as defined in the papers of Dr. Ferry and his associates.

ph 7 and above this slow secondary rise was almost completely inhibited

The effects of ionic strength in sodium and calcium chloride solutions are also shown in Table XXVI experiments 14 to 19 inclusive Rising ionic strength retarded clotting and decreased the turbidity of the clot at a given ionic strength above 0 15 M the calcium ion was more effective than the sodium ion in producing both these modifications The change of turbidity with time for all these solutions is also shown in more detail in Figure 66

The study of the effects of other substances was carried out at constant total ionic strength Therefore if we added an ionic re agent the concentration of sodium chloride added was reduced

EFFECT OF IODIDE AND THIOCYANATE ON FIBRIN CLOTTING

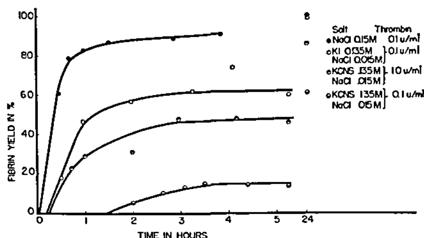


FIGURE 67* Retardation of the clotting process by iodide and thiocyanate ion Note that the ionic strength was 0 15 in all experiments and pH 6 2 — 6 35

correspondingly If we added a neutral molecule like urea the con centration of sodium chloride was held constant at 0 15 M Figure 67 shows the effect of adding iodide or thiocyanate on the rate of fibrin formation Figure 68 shows the corresponding effect of these ions on the opacity of the solution You will see that the effect of both iodide and thiocyanate is to produce a very marked retardation of fibrin formation and to change the properties of the clot so that it becomes more translucent than the control clot at the same pH value The anion of acetyl tryptophan acts in the same fashion

* Figures 67 and 68 present unpublished data of Walter F Lever

The effects of pH on the clotting of human fibrinogen as shown in Table XXVI were in general consistent with the findings of Shulman and Ferry (33) on bovine fibrinogen The rate of fibrin formation increased progressively with increasing pH from 5 8 to 7 or a little above The increase in turbidity with time is a process which could be resolved into at least two major phases During the first few minutes of the reaction just before and just after the onset of clot formation * the rate of increase of turbidity with time was most rapid at pH 7 to 7 3 corresponding to the more rapid rate of clot formation at the higher pH In the later stages of the process however the turbidity rose steadily in the clots formed at lower pH values *corresponding to the secondary formation of thick* fibrin bundles from the primary network of very thin fibrils At

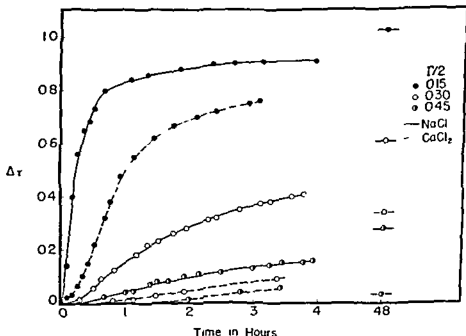


FIGURE 66*† Increment in turbidity as a function of time and ionic strength for clotting mixtures of human fibrinogen (Fraction I) and thrombin for three different concentrations of sodium and calcium chlorides Data for the same solutions are tabulated in experiments 14 19 inclusive of Table XXVI The 48 hour value of $\Delta\tau'$ for the calcium chloride solution was 1 36 at Γ/2 at 0 15 a figure too large to be shown in the diagram

* The clotting time under standard conditions with 0 1 thrombin unit per ml in the solution was approximately four minutes at 23

† Figures 66 and 69 are reprinted by special permission of *J Biol Chem* (In press) *(1951)*

and fibrin in the course of the formation of the fibrin clot Although there clearly must be some sort of range of intermediate polymers in the reaction I felt at that time that a distinct molecular species in between fibrinogen and fibrin had not been identified

However Dr Laki in his fascinating discussion has given clear evidence of such an intermediate — namely in the experiments on treatment with thrombin at an acid pH the molecule which has the same viscosity and the same sedimentation constant as fibrinogen but has the ability to form a polymerized structure very rapidly when the pH is returned to neutrality In the meantime also we at Wisconsin have found an intermediate between fibrinogen and fibrin which is different from the one Dr Laki has demonstrated

This has resulted from studies of the reaction between bovine fibrinogen and thrombin in the presence of reversible inhibitors We have found a number of compounds which are reversible inhibitors of the reaction between fibrinogen and thrombin in the sense that they do not cause permanent damage to either protein but as long as such a compound is present no fibrin clot is formed For example in the case of hexamethylene glycol a concentration of about half molar (at a pH of 6 3 ionic strength 0 45) is enough to prevent clotting even though the mixture stands for days or even weeks But if this reagent hexamethylene glycol is allowed to stand with fibrinogen alone and then dialyzed out again the fibrinogen can react normally with the thrombin and similarly vice versa for incubation with thrombin(35)

Now although fibrinogen and thrombin do not clot in the presence of this compound at a concentration of half molar that doesn't mean that nothing is going on in the system because measurements of sedimentation and viscosity which Dr Shulman and I have made show there is a partial polymerization

The most direct evidence is in the sedimentation diagram in the ultracentrifuge Fibrinogen has a sedimentation constant of about 8 Svedberg units and Armour Fraction I as well as more highly purified preparations from this fraction gives essentially a single peak in the centrifuge If one mixes fibrinogen and thrombin in the presence of hexamethylene glycol after a few hours there is still only one peak in the centrifuge due to fibrinogen but after a day or so a second peak appears(36)

those we have used Its modifying action on clot formation at these low concentrations is most probably due to its very strong tendency to form hydrogen bonds Presumably urea molecules will form such bonds at some of the reactive centers on the fibrinogen or thrombin molecules and thereby retard the process of clot formation

Another well known denaturing agent for proteins is guanidine hydrochloride We have studied its effects also at very low concentrations where it certainly does not have any denaturing action in the ordinary sense Its effects on the clotting process however were profound It was found to resemble urea in that it produced a great retardation of clotting as compared with the control solutions However its effects on the structure of the clot were in the opposite direction to those of urea A clot in a guanidine hydrochloride solution (0 075 M) at pH 7 3 was similar to a clot formed in sodium chloride solutions at pH values nearly one more unit more acid This was true both with respect to the rate of fibrin formation and with respect to the turbidity of the clot (See Table XXVI experiments 9 13 inclusive and compare with experiments 1 8 inclusive) Thus qualitatively the effect of increasing guanidinium ion concentration in the solutions was similar to that of increasing the hydrogen ion concentration This suggests that the guanidinium cation was selectively bound by the protein shifting the properties of the clot in the same direction as if the pH had been lowered by adding acid

These are only a few of the substances that have been studied by ourselves or by other people on their effects on the formation of the fibrin clot They do serve to show among other things how delicately controlled the reaction is and how sensitive it is to apparently small changes in the chemical conditions of clotting One must define conditions with extreme care in order to make sure that one is doing an experiment that can really be reproduced by an experimenter in another laboratory For the most part we know of no experiments in other laboratories to compare with ours except for some of those that Dr Ferry and Dr Shulman have been doing The use of these and other reagents should aid in elucidating the chemical nature of fibrinogen and thrombin and their interaction with one another

I S Wright Dr Ferry?

Ferry Two years ago at this Conference I expressed some skepticism that there was a well-defined intermediate between fibrinogen

species might be Let us consider the various ways in which we could put fibrinogen molecules with the accepted rod like shape together in such a way that the resulting polymer would have three times the sedimentation constant of the unit If they were put together end to end it would take about five thousand of them because the sedimentation constant is so insensitive to length for elongated molecules — that s out of the question because if there were a substantial number of molecules with this length in the solution the viscosity would be enormously higher than observed

If we put them side by side it would take just about four (which is of interest in connection with Dr Laki s recent light scattering measurements) to give the observed sedimentation constant Another possible combination is a hexamer of twice the length and three times the cross section area which would also closely reproduce the observed sedimentation constant I will not attempt to decide between these two possible alternatives at present

Now in addition to this change in the sedimentation pattern some very interesting viscosity changes appear which are a little more complicated to describe As shown in Figure 71 the relative viscosity of the system is initially slightly above 1 This is the viscosity of the solution divided by the viscosity of the solvent As time goes on it climbs up The specific viscosity increases by a factor of twenty or so Eventually it appears to level off This indi

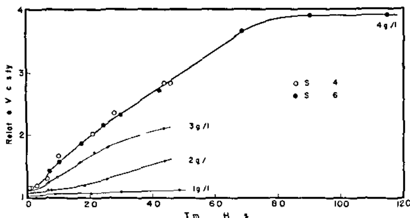


FIGURE 71 Increase in relative viscosity with time in inhibited clotting system fibrinogen (refractionated) 4 gm per l, thrombin 1 unit per ml glycol 0 41 M Small points denote relative viscosities of dilutions to concentrations indicated

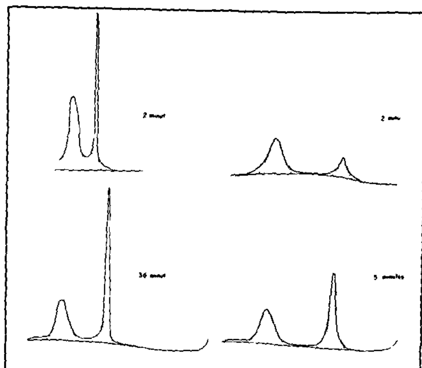


FIGURE 70 Sedimentation diagrams of inhibited clotting systems Upper pair fibrinogen 3 66 gm per l (Fraction I) thrombin, 0 71 unit per ml. glycol 0 36 M reaction time 49 hr (series xxiv) Lower pair fibrinogen 4 gm per l (refractionated) thrombin 1 unit per ml glycol 0 41 M reaction time 51 hr (series 3) The figures denote time elapsed after attainment of full speed in the ultracentrifuge (50 400 r p m)

Figure 70* shows the sedimentation diagram obtained under these circumstances The left peak has the sedimentation constant of fibrinogen 8 5 S and undoubtedly represents unchanged fibrinogen, but a new peak is now here with a sedimentation constant of 24 5 S (values extrapolated to infinite dilution) Now as this system stands for several days the sedimentation constants remain exactly the same We have two peaks which persist for days the sedimentation constants do not change but the fast peak gradually grows at the expense of the slow peak which indicates that fibrinogen is being gradually converted into another molecular species

It is of interest to speculate as to what the size and shape of this

* The investigations described in Figures 70 71 and 72 were supported in part by research grants from the National Institutes of Health Public Health Service by a grant from Eli Lilly and Company and by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation

† Figures 70 71 and 72 are reprinted by permission from *J Phys & Colloid Chem* 55 135 (1951)

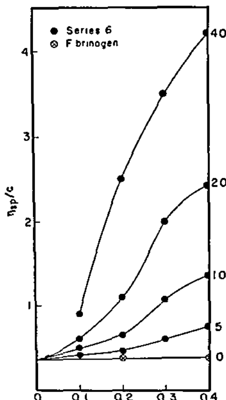


FIGURE 72 Concentration of Fibrinogen in gm./100 ml Reduced specific viscosity of inhibited clotting system plotted against concentration at various times (in hours) interpolated from the data of Figure 71

Alexander May I ask whether your diluent was water saline or what?

Ferry The diluent contains everything in exactly the same proportions with the exception of fibrinogen and thrombin

Alexander What happens if thrombin is kept constant?

Ferry I would not expect any difference because the action of thrombin in the presence of the inhibitor is extremely slow If you make the dilution and quickly make a measurement the thrombin concentration can't make any difference The diluent of course has to be prepared with considerable care because it must have salt and phosphate buffer and hexamethylene glycol in exactly the same proportions as in the protein solution

cates that the molecules are becoming more asymmetrical and the logical thing to suppose is that elongated polymers are being formed

If we want to deduce molecular shape from viscosity measurements what we have to do is to extrapolate the viscosity increment to infinite dilution The usual procedure is to take the relative viscosity which is plotted in Figure 71 subtract 1 from it to give the specific viscosity and divide by the concentration This gives a measure of the ability of the molecule to increase the viscosity of the solvent and this quantity extrapolated to zero concentration is called the intrinsic viscosity from which the deductions can be made regarding molecular shape

If we attempt to do this for our polymerizing system a peculiar thing happens The viscosity drops enormously with dilution The lower curves in Figure 71 represent viscosities of solutions diluted after various time intervals and interpolations at a given time provide the data we would need to extrapolate to give an intrinsic viscosity What we get is shown in Figure 72

The extrapolation for fibrinogen alone which has not been polymerized gives the bottom curve which has a slight positive slope as is normal and specifies the value of the intrinsic viscosity to be 0 34 from which the axial ratio of the fibrinogen molecule can be deduced

Now after a polymerizing system has stood for forty hours if we had a permanently polymerized molecule – a long chain as we thought we would have by this time judging by the magnitude of the viscosity at this point – we would expect again a line of slight positive slope which would extrapolate to an intrinsic viscosity of about 4 But actually the curve falls off so rapidly that by the time we approach zero concentration we are back to the intrinsic viscosity of plain fibrinogen This can hardly mean anything else but that there are long linear aggregates in the solution which dissociate with dilution so that by the time one gets to zero concentration one is left with very short particles

The simplest mechanism which would explain these results is something like this Fibrinogen $\xrightarrow[\text{glycol}]{\text{thrombin}} \Phi \rightleftarrows \Phi n$

Here Φ denotes a certain intermediate which can associate reversibly to form long linear aggregates

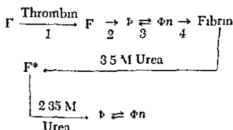
which when we measure its sedimentation constant and its intrinsic viscosity appears to have values for these constants identical with those of fibrinogen In other words this gives additional evidence to supplement Dr Laki's light scattering measurements that the particles which are formed by disruption of the fibrin clot in 3.5 M urea are apparently identical in gross size and shape with the original fibrinogen

Kinsely Would it be possible to take thrombi surgically removed thrombi and run them through this kind of an analytical process and see what proteins are actually present?

Ferry You cannot dissolve it with urea because it is formed physiologically in the presence of calcium It is only a clot from purified fibrinogen and thrombin which you can dissolve in urea

When we have our solution of broken down fibrin in 3.5 M urea we can reduce the urea concentration to 2.35 M keeping everything else constant and we again get our second peak in the ultracentrifuge with a sedimentation constant of about 25 S and again we get our increased viscosity which falls off with dilution and so on

If we try to put all that information together in some sort of reaction scheme I think this is the way it would look



Step 1 produces F an activated fibrinogen This is the one that Dr Laki has in acid solution which has the same molecular dimensions as the original fibrinogen With acid at pH 5 we stop here but in the presence of hexamethylene glycol or urea we go on with Step 2 to our intermediate which has the high sedimentation constant and is a partial polymer with four to six units This is in reversible equilibrium (Step 3) with an end to end polymer and the position of this equilibrium depends on how concentrated the glycol or the urea is If we dialyze out the glycol or the urea the

Although the viscosity drops right off the sedimentation pattern doesn t change very much The fast peak does not disappear so tentatively we identify this with the intermediate P, which can aggregate reversibly to give the long chains responsible for the high viscosity

Now there are two other pieces of evidence for the existence of such elongated polymers One is that the viscosity depends on the rate of shear as one would expect for a system containing long chains Also we took some solutions to Iowa State College where Dr J F Foster has a very good double refraction of flow apparatus patterned after the one Dr Edsall has at Boston Dr Foster and Dr Samsa made flow birefringence measurements on the polymerized systems and found evidence of elongated particles of varying lengths with a mean length perhaps somewhere in the neighborhood of 4000 Å representing a considerable end to end polymerization *of fibrinogen molecules*

More recently Dr Ehrlich and Dr Shulman and I have made measurements on systems containing urea One can substitute urea for hexamethylene glycol as the inhibitor and get almost the same picture – either at pH 6 with 1 M urea or at pH 7 5 with 2 35 M urea Again after a period of hours a new peak appears in the centrifuge with a sedimentation constant of about 25 S and grows at the expense of the fibrinogen peak(39)

Again when the new peak appears in the centrifuge we get a marked increase in viscosity which however falls off with the dilution so that if you try to calculate the intrinsic viscosity you end up *with essentially the intrinsic viscosity of the original* fibrinogen

Edsall In the experiments with urea was the ionic strength also 45?

Ferry No it was 0 15 in this case

Now urea gives us another trick to play because we also have confirmed the observations of Dr Laki Dr Lorand Dr Mihalyi and Dr Edsall that clots of fibrin formed from purified material will dissolve in concentrated urea solutions

We formed clots in cellophane tubes and dialyzed them against urea at a concentration of 3 5 M so that the concentration never rises above that level (*which is desirable because higher concentrations of urea can denature*) Thus we can get a dissolved fibrin

of course due to the rigidity of the clot and one can watch this torque as the function of time and see whether it is necessary to keep applying a torque in order to keep it in twisted position

In a clot from purified fibrinogen and thrombin this torque decays quite rapidly with time In fact in just a few minutes it has dropped to a small portion of its initial value This was astonishing to me because I thought that a clot was a pretty solid structure Apparently the linkages can dissociate or rearrange fairly rapidly This happens by the way both in a so called fine clot and in a coarse clot But if we formed —

Edsall That is in about four or five minutes?

Ferry In a typical case 80 per cent relaxation in ten minutes

Kniseli What is the distance between the two cylinders and what is the force you exerted?

Ferry The force is very small — enough so that the clot is only distorted by a few per cent and by a few per cent I mean that if we imagine a cube cut out of a clot we are deforming the cube in such a way that we are only shoving the top over perhaps 3 per cent That is a very small angle you see so it is not disrupting nor breaking it

Knisely Would it take 100 gm or what?

Ferry I can present that in the form of rigidity The rigidity is of the order of 10^4 dynes per square centimeter That would be for a 1 per cent clot

If one forms a clot in the presence of calcium and serum factor — the stress decays very little

Seegers I want to ask one question in regard to a publication by Kenneth Robbins that was in the *American Journal of Physiology*(39) quite a number of years ago Does that have priority in regard to the observations that a thrombin clot is different from a thrombin plus calcium clot or did someone else make that observation before Robbins did?

Laki As far as I know he studied the solubility of clots in weak acids and alkalies

Ferry That is correct I believe

Ferguson I have two figures which represent experiments of the type presented by Dr Laki and discussed in a current publica

aggregates will go on to form fibrin (Step 4) The details of this step are of course rather obscure

If we put the fibrin into 3 5 M urea we will form fragments which I will symbolize by F because they may not be exactly the same as the original activated fibrinogen Γ although they do have the same molecular dimensions apparently If we now reduce the urea concentration to 2 35 M we get apparently the same polymer Φ which is again in reversible equilibrium with the long aggregates Φ That is the story for today

Warner If you take all the urea out will it go back to fibrin?

Ferry Yes though perhaps I should qualify that by saying that it makes a *coagulum* I think Dr Laki has found that such a coagulum in the electron microscope looks like a fibrin clot So it presumably is not too different from the original fibrin clot

We do not know whether our intermediate is part of a normal clotting process of course because what we have done is to distort the normal clotting process by the inhibitors so that we can build up a high enough concentration of Φ to recognize But when two such widely different compounds as urea and hexamethylene glycol give essentially the same picture it seems to me unlikely that this is an artifact and I think we can assume for the time being that the normal clotting process goes through a stage something like this

If so I wonder if the intermediate is present in any perceptible amount in normal plasma It is a very sticky substance you see since it has a tendency of polymerizing end to end this is a wild idea but perhaps it might have something to do with the stickiness of red cells or platelets However that is pure speculation

Now I have one other item which has nothing much to do with this story but bears on the question of clots which are soluble versus clots which are insoluble in urea

There is a good deal of evidence now that whereas clots formed from purified fibrinogen and thrombin will dissolve in urea those which are formed in the presence of calcium plus an unknown serum factor will not We have some other evidence about the difference between these two types of clots which is concerned with stress relaxation(38)

If you form a clot between concentric cylinders and give the outer cylinder a little twist the inner cylinder experiences a torque

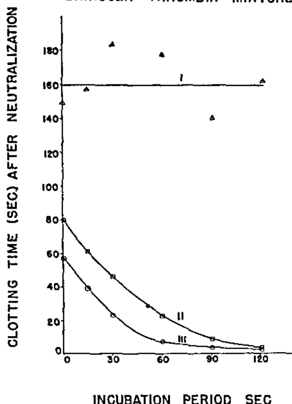


Figure 74

alkaline phosphate but diluted respectively with one half and with one volume of distilled water in the final clotting test Whereas the clotting times of I show no significant change the clotting times in II and III show the "progressive drop and reach a minimum in only a couple of minutes incubation None of the acidified mixtures clotted and the turbidity produced by the acid phosphate acting on the proteins at high salt concentration was very minor If however the acid mixtures were diluted with one or two volumes of water *even without prior incubation* gross flocculation appeared in a few minutes and after an hour or two became a fairly good fibrin gel The better the acidified mixtures clotted in these controls the more striking was the progressive drop in clotting times of samples of similar (lower salt containing) acid mixtures when neutralized after successive periods of incubation

tion(40) Figure 73 shows the clotting times (seconds) obtained with samples of acidified (to pH = 5) phosphate fibrinogen thrombin mixtures when reneutralized (to pH = 6 5) with alkaline phosphate after varying periods of incubation (minutes at 24°C) The over all salt concentration was varied in the three experiments and the specific resistance measurements (in ohm cm) afford a relative indication of this It is significant that at higher ionic strengths (lower specific resistances) as in the top two curves the clotting time fluctuations are within the limits of experimental error throughout the experiment It is only at lower salt concentra tions is in the bottom curve that the clotting times are shorter from the start and drop progressively with incubation in the manner characteristic of the data of Dr Laki s experiments

In Figure 74 the top curve (I) was at high salt concentration very similar to the 59 ohm cm curve (middle) of Figure 69 Curves II and III were obtained simultaneously from exactly the same acid incubated mixture reneutralized with the same amount of

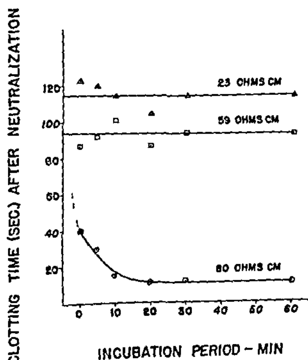


FIGURE 73

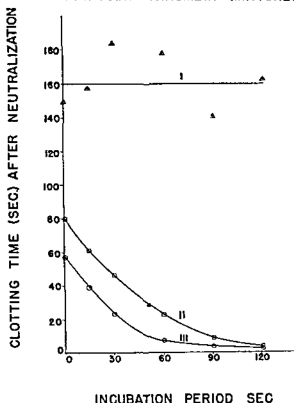


FIGURE 74

alkaline phosphate but diluted respectively with one half and with one volume of distilled water in the final clotting test Whereas the clotting times of I show no significant change the clotting times in II and III show the progressive drop and reach a minimum in only a couple of minutes incubation None of the acidified mixtures clotted and the turbidity produced by the acid phosphate acting on the proteins at high salt concentration was very minor If however the acid mixtures were diluted with one or two volumes of water *even without prior incubation* gross flocculation appeared in a few minutes and after an hour or two became a fairly good fibrin gel The better the acidified mixtures clotted in these controls the more striking was the progressive drop in clotting times of samples of similar (lower salt containing) acid mixtures when neutralized after successive periods of incubation

It seems clear to us that the chief experimental circumstance introduced by Mommaerts and Laki is an excessively high salt concentration long known to be inhibitory to the thrombin fibrin ogen reaction Were it not for the salts present it would be impracticable to carry the acidification to pH = 5 0 because of isoelectric flocculation a process which is much modified by salt concentration(41)

The interpretation of all this work in our opinion must be in terms of delay of the thrombin fibrinogen reaction by unfavorable ionic atmosphere(42) Unfavorable conditions lengthen the latent period before visible fibrin formation but do not necessarily inhibit the polymerization processes completely Thus it is probable that the restoration of favorable conditions in which lowering of salt content is perhaps even more important than pH enable the clotting reaction to carry on to completion the more rapidly the more it has been able to proceed through the earlier polymerization phases in the extended latent period I do wonder about the technical adequacy of the evidence (e g viscosimetry and ultra centrifugation data) upon which Dr Laki bases his conclusion as to absence of polymerization (of fibrinogen) under these complex experimental conditions

Laki I agree that the conditions are unfavorable but I make it unfavorable on purpose I do not pretend that it is only the pH that prevents the clotting the salt probably has some effect too It would not be possible to keep the fibrinogen in solution at this pH without the salt

Link As a chemist by hobby I am interested in seeing carbo hydrates mixed up with blood coagulation Did I understand cor rectly that you said you have isolated and identified galactose as a part of the fibrinogen?

Laki No I estimated the carbohydrate content with a color test and expressed its amount as galactose I have no idea what kind of carbohydrate it might be

Link And you also found it in fibrinogen as I understand and then you also had a carbohydrate left in supernatants which is dialyzable May I suggest that you look for an amino sugar?

Laki I found about 17 per cent less carbohydrate in the super natant after dialysis This difference is not large enough to make definite conclusions Certainly large parts of the carbohydrates in

the supernatant are undialyzable probably bound to protein The carbohydrate could be amino sugar as well

Knisely I have something which may cost you your life if you don t let me mention it I feel this way – somebody identified clotting with thrombosis simply because in Greek the word thrombus means clot Here these gentlemen talk about different methods for dissolving different types of proteins etc for solvents – urea and guanidine and other substances can be used And now with surgeons removing thrombi during surgery and with thrombi having "necks and tails it is time that chemists began cutting these definable parts of large thrombi into pieces dissolving them in some way and seeing what chemicals the parts of thrombi are really made out of There is no reason why we should be going on with this simple morphological indentification when the anatomical parts can be separated and each examined chemically The proteins found in thrombi should also be examined by immune chemists Special "immune proteins may be participating in the formation of thrombi

I S Wright We will defer this problem until next year Meanwhile the chemists can contemplate what they are going to do with the heads bodies and tails of these clots

Knisely It is time to do it

I S Wright In that case will you please identify the source of your thromboplastin thrombin fibrinogen and other components so that all may understand exactly what you are using

REFERENCES

1 FERRY J D Protein Gels *Advances in Protein Chemistry* Vol IV Anson M L and Edsall J T Editors New York Academic Press Inc 1948

2 LAKI K and LÓRAND L On the solubility of fibrin clots *Science* 108 *280 (1948)*

3 LÓRÁND L A study on the solubility of fibrin clots in urea *Hungarica Acta Physiol* 1 *192 (1948)*

4 LAKI K and MIHALYI E Action of thrombin on iodinated fibrinogen *Nature* 163 *66 (1948)*

5 LÓRAND L Fibrin clots *Nature* 166 *694 (1950)*

6 MIHÁLYI E The urea denaturation of fibrinogen 1 Kinetic aspects *Acta Chem Scandinav 4 317 (1950)*

7 LAKI K Redoxpotential and resonance energy of certain quinones *Stud Inst Med Chem Univ Szeged* 2 49 (1942)

8 LAKI K and CSOKAN P Zur Structur der Proteine *Zeitschr physik Chem* A190, 278 (1942)

9 FERRY J D and MORRISON P R Preparation and properties of serum and plasma proteins The conversion of human fibrinogen to fibrin under various conditions *J Am Chem Soc* 69 388 (1947)

10 FERRY J D and SCHULMAN S The conversion of fibrinogen to fibrin II Influence of pH and ionic strength on clotting time and clotopacity *J Phys & Colloid Chem* 54 66 (1950)

11 LAKI K and MOMMAERTS W F H Transition of fibrinogen to fibrin as a two step reaction *Nature* 156 664 (1945)

12 STEINER R F and LAKI K Light scattering studies on fibrinogen preliminary remarks *J Am Chem Soc* 73 882 (1951)

13 COHN D J *et al* Preparation and properties of serum and plasma proteins IV A system for the separation into fractions of the protein and lipoprotein components of biological tissues of fluids *J Am Chem Soc* 68 459 (1946)

14 LYONS R N Thiol vitamin K mechanism in the clotting of fibrinogen *J Exper Biol & Med Sc* 23 131 (1945)

15 BAGDY D *et al* The reducing properties of fibrinogen the role of SH groups in the clotting of fibrinogen *Hungarica Acta Physiol* 1 197 (1948)

16 EAGLE H and HARRIS T N Studies in blood coagulation coagulation of blood by proteolytic enzymes (trypsin papain) *J Gen Physiol* 20, 543 (1937)

17 EAGLE H Coagulation of blood by snake venoms and its physiologic significance *J Exper Med* 5 613 (1937)

18 FERGUSON J H and RALPH P H Ninhydrin crystalline papain and fibron clot *Am J Physiol* 138 618 (1945)

19 DYCKERHOFF H and GIGANTE D Über das Vorkommen von Phyto Thrombin in Papain (Beziehungen das Papains zum Blutgerinnungssystem) *Biochem* 7 304 334 (1940)

20 GUEST M M and WARE A G Dissolution of fibrin clots in the presence of concentrated purified thrombin *Federation Proc* 9 53 (1950)

21 HAWN C V Z and PORTER K P The fine bone structure of clots formed from purified bovine fibrinogen and thrombin a study with the electron microscope *J Exper Med* 86 286 (1947)

22 HALL C E Electron microscopy of fibrinogen and fibrin *J Biol Chem* 179 857 (1949)

23 ONCLEY J L SCATHARD G and BROWN A Physicochemical characteristics of certain of the proteins of normal human plasma *J Phys & Colloid Chem* 51 184 (1947)

24 Edsall J T Foster J F and Scheinberg H Studies on double refraction of flow III Human fibrinogen and fraction 1 of human plasma *J Am Chem Soc 69 2731 (1947)*

25 Nanninga L B Investigations on fibrinogen and thrombin the second phase of blood coagulation Thesis Univ of Amsterdam 1947

26 ——— Preparation photo electric determination molecular weight and viscosity formol titration before and after clotting *Arch Neerlandaises de Physiol* 28 *241 (1946)*

27 Mihályi E and Lórand L The mechanism of clotting of fibrinogen with formaldehyde and quinone Interactions between formaldehyde and thrombin *Hungarica Acta Physiol* 1 *18 (1948)*

28 Waugh D F and Livingstone B J Clotting time and reaction velocity in the interaction of bovine fibrinogen and thrombin *Science* 113 *121 (1951)*

29 ——— Kinetics of the interaction of bovine fibrinogen and thrombin *J Phys & Colloid Chem* To be published

30 Morrison P R Edsall J T and Miller S G Preparation and properties of serum and plasma proteins XVIII The separation of purified fibrinogen from fraction I of human plasma *J Am Chem Soc* 70 *3103 (1948)*

31 Edsall J T and Lever W F *J Biol Chem (1951) (in press)*

32 Morrison P R Preparation and properties of serum and plasma proteins some factors influencing quantitative determination of fibrinogen *J Am Chem Soc 69 2723 (1947)*

33 Shulman S and Ferry J D The conversion of fibrinogen to fibrin II Influence of pH and ionic strength of clotting time and clot opacity *J Phys & Colloid Chem* 54 *66 (1950)*

34 Scatchard G Scheinberg I H and Armstrong S H Physical chemistry of protein solutions IV The combination of human serum albumin with chloride ion *J Am Chem Soc* 72 *535 (1950)*

35 Ferry J D and Shulman S The conversion of fibrinogen to fibrin I Influence of hydroxyl compounds on clotting time and clot opacity *J Am Chem Soc* 71 *3198 (1949)*

36 Shulman S and Ferry J D The conversion of fibrinogen to fibrin III Sedimentation and viscosity studies on clotting systems inhibited by hexamethylene glycol *J Phys & Colloid Chem* 55 *135 (1951)*

37 Shulman S Ehrlich P and Ferry J D An intermediate in the conversion of fibrinogen to fibrin *J Am Chem So* 73 *1388 (1951)*

38 Ferry J D Miller M and Shulman S Effect of calcium on rigidity and stress relaxation of fibrin clots *Federation Proc* 10 *182 (1951)*

39 ROBBINS K C Study on conversion of fibrinogen to fibrin *Am J Physiol* 142 581 (1944)

40 BAYLES P W FERGUSON J H and MUEHLKE P H Mechanisms involved in fibrin formation *J Gen Physiol* 34 493 (1951)

41 FERGUSON J H Flocculation maximum (pH) of fibrinogen and some other blood clotting reagents (Relative turbidimetry with Evelyn photoelectric colorimeter) *J Gen Physiol* 25 607 (1942)

42 FERRY J D Consideration of the structure and mechanism of formation of the fibrin clot *Blood Clotting and Allied Problems* Flynn J E Editor Trans Second Conf New York Josiah Macy Jr Foundation 1949 (p 84)

POLAROGRAPHIC STUDIES OF THE FIBRINOGEN FIBRIN REACTION*†

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IN 1945 LYONS(1) proposed a thiol vitamin K scheme to explain the transformation of fibrinogen to fibrin Lyons claimed that a) fibrinogen A is changed to fibrinogen B by the action of thrombin and in the process some undetectable thiol groups of fibrinogen A are "liberated" and make their appearance in fibrinogen B b) vitamin K is able to transform fibrinogen B to fibrin in which the sulphydryl groups are oxidized to the disulphide form c) fibrinogen A and B can be distinguished on the basis that A can be precipitated by sodium chloride from fresh plasma while B can be obtained from aged plasma treated with $Ca (PO_4)$ Lyons based his conclusions in part on some polarographic investigations and it is with this aspect of his work that this paper is concerned

The polarographic method is an electrolytic process which uses a mercury drop electrode in conjunction with a reference electrode usually a saturated calomel half cell The mercury drop electrode is immersed in the solution under investigation while the calomel half cell is connected to the solution by a suitable salt bridge The record called a polarogram consists of a current voltage curve This is obtained by recording the changes in the current passing through the cell while the applied potential is progressively increased(2) In the presence of ammonia and cobalt proteins give a polarographic record unlike that of any other type of compound(3)

In the work we wish to report a conventional type of circuit was used employing a Leeds and Northrop Speedomax recorder which was adapted for the measurement of current A motor driven Heli pot potentiometer was used as the voltage divider The capillary consisted of a length of Corning marine barometer tubing which had the following characteristics With an apparent pressure head

* This material was prepared for the Conference but shortage of time precluded the detailed presentation given here

† This research was supported by a grant from the Life Insurance Medical Research Fund to Dr L B Jaques

of 55 cm Hg m = 1 73 mg/sec and t = 4 07 sec when the capillary was immersed in distilled water on open circuit The capillary constant was 30 92 cm sec mg^{-1}(4)

Typical polarograms of a protein are shown in Figure 75 using insulin The first polarogram was obtained by electrolyzing a solution of 0 001M cobaltic chloride, and 0 1M ammonium chloride A small step or wave can be seen This is due to the reduction of bivalent cobalt The peaked maximum which precedes the wave may be ignored The second record shows the effect of adding protein to such a solution The cobalt wave is still present but it is masked by the protein response If alkali is added to the electrolyte as was done before each of the remaining polarograms was recorded the shape of the curve is altered If the electrolysis is sufficiently prolonged the characteristic protein polarogram exhibits three waves which follow the cobalt wave (One typical record has been marked with an asterisk) We refer to these as Waves I II and III and you can see that as the pH is raised by this procedure Waves I and III tend to disappear whereas Wave II persists to high pH values (pH 12 and 13) For the remainder of this discussion no further mention will be made of Wave III because Wave III of the fibrinogen polarogram is not as well defined as that of some other proteins and it is difficult to measure with accuracy

At constant pH the height of a given wave varies with the protein concentration Figure 76 shows results obtained by us for Wave II using fibrinogen This curve is typical of proteins At low concentration adsorption on the wall of the vessel results in abnormally small waves while at high concentration other factors are involved which tend to depress the wave In between these two extremes the concentration curve usually conforms to a Langmuir adsorption isotherm(6) When the concentration of fibrinogen was kept constant and the pH of the electrolyte varied the results shown in Figure 77 were obtained

Similar results have been obtained with all proteins tested The curve is not well understood at present but the magnitudes of its various parts and the position of the hump on the pH scale are characteristic of the protein

The magnitude of the flat portion of this curve (u) was determined for a number of crystalline proteins (insulin chymotrypsin chymotrypsinogen pepsin and egg albumin) From analytical data

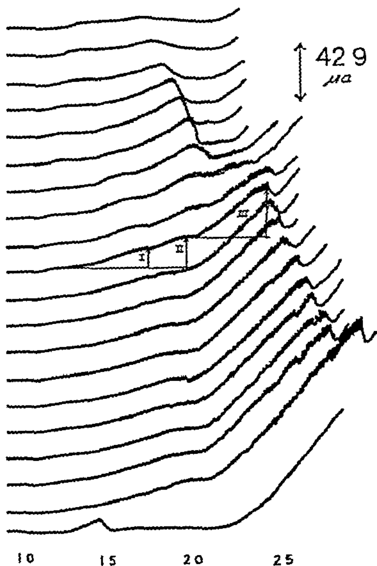


Figure 7a The effect of electrolyte pH on the polarogram of a protein Temperature 10 C

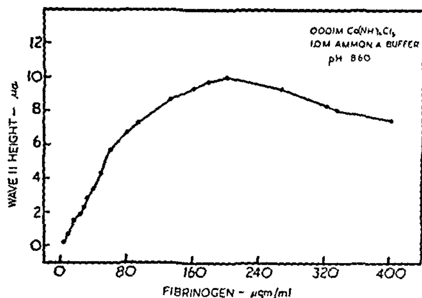


FIGURE 76 The relationship between protein concentration and the magnitude of wave II Beef fibrinogen prepared according to Jaques(5)

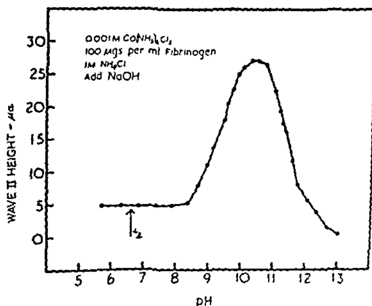


FIGURE 77 Variation in the magnitude of wave II with electrolyte pH Fibrinogen concentration in the cell was 0 1 mg per ml

reported in the literature the total potential sulphydryl content (cysteine + 2 × cystine) was calculated for each protein As shown in Figure 78 there appears to be a very significant correlation between total potential RSH and the quantity i_l This is the first time that a *quantitative* correlation has been found between the heights of the polarographic waves of different proteins and any aspect of their amino acid content

Typical protein waves have been obtained only with proteins which contain cysteine and/or cystine and on the basis of this and other evidence Brdicka postulated the following reactions in order to explain the first two polarographic protein waves

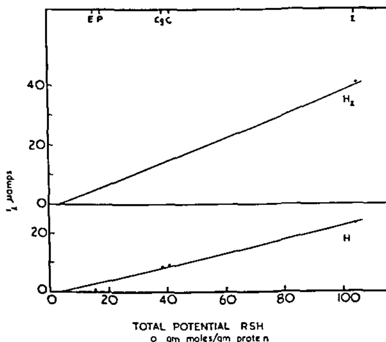
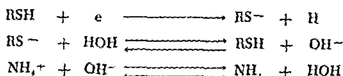
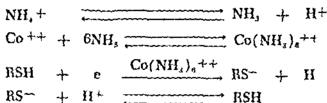


FIGURE 78 The relationship between i_l and the total potential sulphydryl content of the protein E = egg albumin P = pepsin Cg = Chymotrypsinogen, I = insulin All proteins were crystalline Insulin from Connaught Laboratories Toronto and all other from Armour and Co Chicago Protein concentration in each case 0 07 mg per ml i_l = magnitude of the flat portion of the wave height — pH curve



$$RSH + e \longrightarrow RS^- + H$$
$$RS^- + HOH \rightleftharpoons RSH + OH^-$$
$$NH_4^+ + OH^- \rightleftharpoons NH_3 + HOH$$

In our view these reactions should be modified as follows



$$NH_4^+ \rightleftharpoons NH_3 + H^+$$
$$Co^{++} + 6NH_3 \rightleftharpoons Co(NH_3)_6^{++}$$
$$RSH + e \xrightarrow{Co(NH_3)_6^{++}} RS^- + H$$
$$RS^- + H^+ \rightleftharpoons RSH$$

This places greater emphasis on the importance of the bivalent cobalt ammonia complex According to this concept cobalt in the presence of ammonia forms the bivalent cobalt hexamine complex which coordinates with the sulphur of the sulphydryl group thereby allowing hydrogen to be split off and reduced at the electrode The sulphur anion regenerates sulphydryl groups by accepting a hydrogen ion from the buffer The sulphydryl group may then participate in the electrode reaction once more To use an analogy the protein is acting as a hydrogen carrier

It is usually considered that denaturation of a protein liberates sulphydryl groups Since mild heating or short exposure to ultra violet light causes a large increase of polarographic protein waves the polarograph is a very sensitive indicator of sulphydryl "liberation

We will now consider the work of Lyons in the light of the principles outlined and by an examination of Lyons polarographic data and our own experimental data Figure 79 shows polaro graphic curves presented by Lyons(1) as proving his hypothesis Referring to this figure Lyons states Figure 4* shows the increase in number of thiol groups when 0 1 ml of thrombin is added to fibrinogen A solution (0 5 mg per 100 ml) in a supporting electrolyte of 0 001 N cobaltous chloride 1 N ammonia and 1 N ammonium chloride E was the polarogram obtained with cobalt and buffer alone and A when fibrinogen A was added to the ammoniacal cobalt solution Lyons states that fibrinogen A can be distinguished from a typical thiol curve by the absence of maxima (The maxima

* Figure 79 in this publication

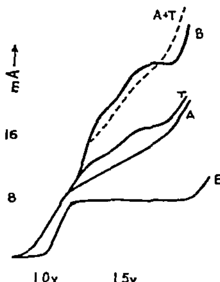


FIGURE 79 Polarograms of fibrinogen thrombin and their mixture Increase in thiol groups of fibrinogen A upon the addition of small amounts of thrombin

A 0 5 mg fibrinogen A per 100 ml
B 0 1 ml dilute thrombin added to A
T 0 1 ml dilute thrombin
E supporting electrolyte

Reproduced with modification from Lyons R N *Australian J Exper Biol & Med Sci* 23 131 (1945) pH approximately 9 2

he refers to are the humps of the typical protein polarogram such as are seen in the remaining polarograms) His fibrinogen A polarograms are certainly atypical and unlike any we have seen with any protein including fibrinogen prepared by Lyons own method or by the method of Jaques(5) or Seegers(7) or the Harvard low temperature fractionation represented by bovine Fraction I of Armour T represents the record obtained with thrombin alone This is a typical protein polarogram B represents the effect when both thrombin and fibrinogen are present in the cobalt ammonia system The magnitudes of the waves in this record are larger than the record of fibrinogen A alone and Lyons has concluded that this demonstrates a liberation of sulphydryl groups in fibrinogen A with the formation of fibrinogen B He drew this conclusion because he had previously observed that a given concentration of his fibrinogen B (from aged blood treated with Ca (PO)₂ always yielded larger waves than the same concentration of fibrinogen A

It is obvious that Lyons had neglected the current which the protein in the thrombin preparation contributed to curve B To allow for this approximately, we can add the thrombin and fibrinogen A current and on plotting this we obtain the broken line A + T On this basis we conclude that little evidence has been presented of thrombin modifying the polarographic behaviour of fibrinogen and therefore there is no basis for Lyons's conclusion that thrombin liberates sulphydryl groups of his fibrinogen A

We have repeated Lyons work following his instructions closely and have obtained records which were similar to his except that our fibrinogen A polarograms always showed two typical protein waves The results of one experiment are shown in Figure 80 In order to magnify and make visible any possible increase the original polarograms have not been reproduced but instead the height of Wave II has been plotted on an enlarged scale The height of Wave II was chosen as the most representative value for the polarogram of fibrinogen The addition of fibrinogen A to the solution produced a wave height of about 8 microamperes in this experiment The addition of small quantities of thrombin caused the wave to increase From control experiments it was found that the added thrombin itself would contribute to the height of the original fibrinogen wave according to the dotted line The increases in wave height are within the experimental error of the method

The polarographic work which has been discussed was handicapped by the fact that because of its low potency comparatively large quantities of thrombin had to be added to the solution thereby necessitating correction for the current contributed by the thrombin Furthermore *the fibrinogen solution prepared by Lyons technique* undoubtedly contained a high proportion of other proteins Recently we have been able to neglect the thrombin correction for Dr Seegers very kindly gave us a sample of thrombin which had a potency of approximately 800 units per milligram of dry weight (Dr Seegers assay) This meant that we could add to our cell a quantity of thrombin which under appropriate conditions would ensure clotting but which would not cause a detectable polarographic current of its own The fibrinogen solution was prepared by the method of Jaques(5) and was about 94 per cent clottable

Figure 81A shows the height of Wave II at various time intervals following the addition of fibrinogen to the electrolytic cell The wave height falls slowly with time B shows the effect of adding

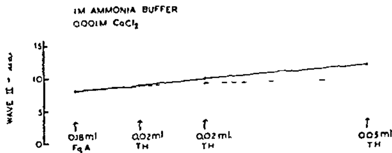
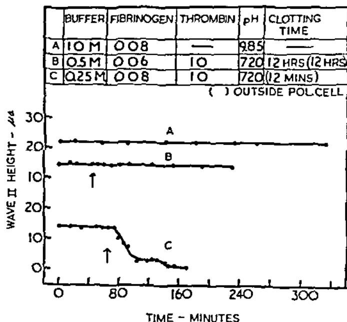


FIGURE 80 Th effect of added thrombin on the height of polarographic wave II of fibrinogen A Solid line — solution contained both fibrinogen A and thrombin Broken line — sum of fibrinogen A current and current contributed by thrombin alone Fibrinogen prepared according to Lyons(1) and thrombin obtained from the Harvard low temperature laboratory



	BUFFER	FIBRINOGEN	THROMBIN	pH	CLOTTING TIME
A	1.0 M	0.08	—	9.85	—
B	0.5 M	0.06	1.0	7.20	12 HRS (12 HRS)
C	0.25 M	0.08	1.0	7.20	(12 MINS)

FIGURE 81 Variations in the height of wave II of fibrinogen in the presence and absence of thrombin Arrow indicates the time that thrombin was added Thrombin supplied by Dr Seegers Fibrinogen prepared according to Jaques(5)

some of Dr Seegers thrombin to this solution As in Lyons experiments the pH and ionic strength were such that actual clot formation did not take place in the cell during the period of observation There is no significant change in the fibrinogen wave height follow

ing the introduction of thrombin In the experiment represented at the bottom C the pH and ionic strength were such that fibrin formation took place in the cell and it can be seen that the magnitude of the fibrinogen wave decreased as soluble protein was removed from the solution There was no detectable increase in the wave height to suggest liberation of fibrinogen sulphydryl groups prior to fibrin formation This last experiment suggests that this method might be used to study the kinetics of the clotting reaction and Mr Schucher has started some work along these lines

Cobalt concentration	0.005M	in 490 and 536
	0.002M	in 843
	0.001M	in all others
Fibrinogen concentration	0.1 mg/ml	in 615
	0.08 mg/ml	in 312 322 414 490 557 and 843
	0.06 mg/ml	in 536 582 596
	0.05 mg/ml	in 640
	0.03 mg/ml	in 512
	0.02 mg/ml	in 497 530 875

Table XXVII summarizes the results of a number of polarographic experiments similar to those in Figure 81 A relatively wide range of buffer concentrations was used The fibrinogen concentration was varied from 0.02-0.08 mg per ml and thrombin (Seegers) from 0.1 to 1 units per ml The pH was varied from 7.2 to 9.65 The thrombin was added at various times after the fibrinogen was first introduced and in each case the clotting time was recorded in control tubes outside the cell Clotting was observed in the electrolytic cell at about the same time In no experiment was there any evidence of any increase in the wave height of the fibrinogen polarogram following the addition of thrombin and we therefore conclude that the liberation of the sulphydryl groups of fibrinogen by thrombin has not been demonstrated by either Lyons work or our own

CONCLUSION

An examination of the published polarograms of Lyons reveals that the sum of the polarographic currents of thrombin and of fibrinogen A approximate the current due to the electrolysis of the mixture of these two preparations We do not agree that his polarograms indicate the liberation" of fibrinogen thiol groups by thrombin We have repeated Lyons preparative and polarographic techniques Our results resembled those of Lyons and we have drawn the same negative conclusion from them The polarograms of relatively *pure* fibrinogen preparations have been examined

TABLE XXVII

Summary of Experimental Conditions and Results of Polarographic Experiments

Expt No	Buffer conc (Molar)	Thrombin Conc (Units/ml)	pH	Time Thrombin added (min)	Duration of Expt after thrombin added (min)	Clotting time of Control	Wave Height—a	
							Before Thrombin	After Thrombin
312	1 0	0	9 85	—	—	5 5 hr	—	—
322	0 5	1 0	9 65	0	240	30 36 "	—	—
414	0 5	1 0	8 20	42	258	18 20 "	9 55	9 35
536	0 5	1 0	7 20	46	184	12 "	14 60	14 60
582	0 30	1 0	8 50	50	105	4 "	9 56	8 15
557	0 30	1 0	9 20	136	156	8-10 "	13 80	13 78
596	0 25	1 0	8 90	20	140	5 "	8 15	8 15
530	0 25	0 2	8 57	43	148	4 6 "	2 08	2 07
497	0 25	0 1	9 45	15	120	16 "	4 57	4 37
512	0 15	0 1	9 04	54	52	1 "	5 43	5 45
843	0 10	1 0	7 20	35	95	3 min	7 05	2 28
873	0 10	0 2	6 60	38	68	8 "	1 49	1 49
640	0 10	0 1	8 10	33	138	10	3 97	4 17
615	0 10	1 0	8 75	19	112	8 "	7 53	7 53
490	0 25	1 0	7 20	62	108	12 "	13 75	13 80

before and after the addition of a potent thrombin preparation under a variety of conditions of pH buffer concentrations fibrinogen concentration and thrombin concentration In some experiments the conditions were such that clot formation did not occur in the electrolysis cell while in other experiments clot formation was observed In no experiment were the fibrinogen waves enhanced by the addition of thrombin and we found no evidence of a thrombic liberation of fibrinogen thiol groups by this procedure When clotting occurs in the electrolytic cell the protein current falls with time as fibrinogen is removed from solution An attempt is being made to use this procedure to study the kinetics of the thrombin fibrinogen reaction

I S Wright This concludes the Fourth Conference on Blood Clotting and Allied Problems

REFERENCES

1 LYONS R N Thiol vitamin K mechanism in clotting of fibrinogen *Australian J Exper Biol & Med Sc* 23 *131 (1945)*

2 KOLTHOFF I M and LINGANE J J *Polarography* New York Interscience Publishers Inc 1941

3 BRDICKA R Polarographic studies with the dropping mercury cathode XXXI New test for proteins in the pres nce of cobalt salts in ammoniacal solutions of ammonium chloride *Collection Czechoslov Chem Communications* 5 *112 (1933)*

4 MULLER O H The characterization of capillaries used for dropping mercury electrodes in polarographic studies *J Am Chem Soc* 66 *1019 (1944)*

5 JAQUES L B The reducing properties of fibrinogen *Biochem J* 37 *344 (1943)*

6 BRDICKA R Polarographische Studien mit der Tropfenden quicksilberkathode LXXXV Uber Grenzstrome der katalysierten Elektrodenvorgange *Collection Czechoslov Chem Communications* 11 *614 (1939)*

7 WARE A G GUEST M M and SEEGERS W H Fibrinogen With special refe n e to its preparation and certain properties of the product *Arch Biochem* 13 *231 (1947)*